ΠΑΝΕΠΙΣΤΗΜΙΟ ΚΡΗΤΗΣ
ΤΜΗΜΑ ΙΑΤΡΙΚΗΣ

UNIVERSITY OF CRETE
FACULTY OF MEDICINE

Πρόγραμμα Μεταπτυχιακών
Σπουδών στις Νευροεπιστήμες

Graduate Program in
Neuroscience

"Investigation of the cannabinoid system in healthy and ischemic retina"

Sofia Papadogkonaki

Supervisor
Dr. Kyriaki Thermos
Professor of Pharmacology

September, 2016

Department of Pharmacology

Faculty of Medicine

University of Crete

Supervisor

Dr. Kyriaki Thermos

Professor of Pharmacology

Members of Committee

Dr. Kyriaki Thermos, Professor of Pharmacology

Dr. George Liapakis, Associate Professor of Pharmacology

Dr. Ioannis Charalambopoulos, Assistant Professor of Pharmacology

Acknowledgements

I would like to express my deepest appreciation to my supervisor, Professor Kyriaki Thermos, for her continuous support, her guidance, patience, and inspiration.

Besides my supervisor, I would like to thank the present members of the Pharmacology lab, Niki Mastrodimou, Ruth Iban Arias and Mirto Fothiadaki, for the wonderful cooperation and for both technical and scientific advice that helped me to accomplish my Master thesis. A pleasant work environment is very important in order to be productive and efficient.

In addition, I would like to thank Despina Kokona and Silvia Lisa Ferrer, who despite the fact they are past lab members, their advice and guidance is still essential for my work. Despina Kokona was the one who taught me the laboratory techniques that I have used, and despite her workload, she was always available for my questions.

I would also like to thank my friends, who supported me and have been so patient listening to my problems and thoughts. Last but not least, I thank my family, my parents Giannis and Mary and my sister Christina, who supported me spiritually throughout these years and taught me how to be strong in order to fulfill my ambition.

Abstract

Cannabinoids have been shown to protect the retina, in different models of neurotoxicity via activation of the CB1 receptor (CB1R) and its downstream PI3K/Akt signaling pathway. Chronic exposure of the synthetic cannabinoid HU-210 induced the downregulation of the CB1R in several brain regions. The aim of this study was to investigate whether subchronic or chronic administration of HU-210 leads to downregulation of the CB1R in healthy and ischemic rat retina.

Sprague-Dawley rats were administered with HU-210 (25, 50 and 100 μg/kg, i.p) daily for 4 or 14 days (subchronic or chronic administration). To study the acute effects of HU-210, rats were injected with vehicle for 13 days and with HU-210 on the 14th day. Immunohistochemical studies and western blot analysis were performed to examine the downregulation of the CB1R and the phosphorylation of Akt protein. Acute and subchronic effects of HU-210 on CB1 receptor expression and on the neuroprotection of amacrine cells in the in vivo model of AMPA excitotoxicity were examined.

CB1R immunoreactivity is localized in the ganglion cell, inner plexiform, inner nuclear and outer plexiform layers. A decrease of CB1R immunoreactivity was observed after 4 or 14 days of HU-210 (50 and 100 μg/kg, i.p.) administration. Similar results were obtained with western blot analysis. Acute administration of HU-210 (100μg/kg) decreased the CB1R immunoreactivity. This dose also led to the reduction of Akt phosphorylation after chronic treatment. Co-administration of HU-210 (10^{-6} M) with AMPA (8.4mM) afforded neuroprotection to retinal amacrine cells against AMPA excitotoxicity, while subchronic administration of HU-210 (50μg/kg, i.p) attenuated this effect and decreased CB1R immunoreactivity.

This study provides novel information regarding the chronic effect of HU-210 in healthy rat retinas and in animal models of retinopathy. HU-210, caused a time- and dose-dependent downregulation of the CB1R in rat retina and a reduction in the activation of PI3K/Akt signalling pathway. Down regulation of the CB1R was also observed after subchronic administration of HU-210, in the AMPA excitotoxicity model, leading to the loss of neuroprotection to amacrine cells. These results suggest that HU-210 does not appear to be the appropriate therapeutic for chronic retinopathies.

Περίληψη

Τα ενδογενή και συνθετικά κανναβινοειδή προστατεύουν τον αμφιβληστροειδή σε διάφορα μοντέλα νευροτοξικότητας μέσω της ενεργοποίησης του υποδοχέα CB1 (CB1R) και του σηματοδοτικού μονοπατιού PI3K/Akt. Ωστόσο, η χρόνια χορήγηση του συνθετικού κανναβινοειδούς HU-210 επάγει τη μειορρύθμιση του CB1R στον εγκέφαλο. Σκοπός της παρούσας μελέτης ήταν η διαλεύκανση της επίδρασης της υποχρόνιας και χρόνιας χορήγησης του HU-210 στη ρύθμιση του CB1R στον υγιή και ισχαιμικό αμφιβληστροειδή.

Sprague-Dawley επίμυες υποβλήθηκαν σε καθημερινή ενδοπεριτοναϊκή χορήγηση (i.p.) του HU-210 (25, 50, 100 μg/kg) για 4 ή 14 ημέρες (υποχρόνια ή χρόνια χορήγηση, αντίστοιχα). Για τη μελέτη της οξείας χορήγησης του HU-210 πραγματοποιήθηκε χορήγηση του εκδόχου για 13 ημέρες ενώ το HU-210 ενέθηκε την 14^{η} ημέρα. Η επίδραση της οξείας και υποχρόνιας χορήγησης του HU-210 μελετήθηκε και στο πειραματικό μοντέλο της AMPA διεγερτοτοξικότητας.

Στον υγιή αμφιβληστροειδή, η έκφραση του CB1 υποδοχέα εντοπίζεται στις στοιβάδες: γαγγλιακή, έσω δικτυωτή, έσω πυρηνική και έξω δικτυωτή. Ανοσοϊστοχημικές μελέτες και μελέτες ανοσοαποτύπωσης έδειξαν ότι παρατηρείται μείωση στην έκφραση του CB1R έπειτα από χρόνια ή υποχρόνια χορήγηση του HU-210 (50 και 100 μg/kg), όπως κι έπειτα από οξεία χορήγηση του σε δόση 100 μg/kg. Επιπλέον, χρόνια χορήγηση του HU-210 (100 μg/kg) προκάλεσε μείωση της φωσφορυλίωσης της πρωτεΐνης Akt. Η συγχορήγηση του HU-210 (10^{-6} M) και AMPA (8.4 mM) παρείχε νευροπροστατευτική δράση στα βραχύινα κύτταρα του αμφιβληστροειδή έναντι της AMPA διεγερτοτοξικότητας. Αντιθέτως, η υποχρόνια χορήγηση του HU-210 (50 μg/kg, i.p.) δε μείωσε τις τοξικές δράσεις του AMPA, ενώ προκάλεσε μείωση της έκφρασης του CB1R.

Η παρούσα μελέτη παρέχει σημαντικές πληροφορίες σχετικά με τις δράσεις της χρόνιας χρήσης του HU-210 στον υγιή αμφιβληστροειδή και σε μοντέλο αμφιβληστροειδοπάθειας. Το HU-210 προκαλεί χρονο-εξαρτώμενη και δοσο-εξαρτώμενη μειορρύθμιση του CB1R και μείωση της ενεργοποίησης του σηματοδοτικού μονοπατιού PI3K/Akt. Μειορρύθμιση του υποδοχέα παρατηρείται κι έπειτα από υποχρόνια χορήγηση του HU-210 σε μοντέλο αμφιβληστροειδοπάθειας, οδηγώντας στη μείωση της νευροπροστατευτικής δράσης του. Τα αποτελέσματα αυτά προτείνουν ότι το HU-210 δεν θα είναι αποτελεσματικό στην θεραπεία χρόνιων αμφιβληστροειδοπαθειών.

Contents

1. Introduction

Vision is the most fundamental of our senses. The human eye consists of different parts, which are all important for perceiving a good image. However, the retina is the most vital part for vision, as it is responsible for the captivation of the outer world's images. The retina is considered a part of the central nervous system (CNS) that transforms light to electric signals in order to transmit the visual information to the brain.

Ischemic conditions that are characterized by the insufficient supply of oxygen and nutrients to the retina can lead to retinal cell death (neurodegeneration) and/or neovascularization. These conditions underlie the development of several retinopathies that lead to suboptimal visual acuity and even to blindness. To date, there are not many efficacious drugs for the treatment of retinopathies, as most pharmacologic approaches target the neovascularization and not the ischemic-induced retinal cell death. Therefore, the study of the neuroprotective properties of new agents that may lead to new therapeutics for the treatment of retinopathies is essential. Most importantly, the efficacy of novel neuroprotectants after chronic administration should be examined prior to their characterization as novel therapeutics for retinal diseases that are classified as chronic pathological conditions.

1.1 The anatomy of the eye

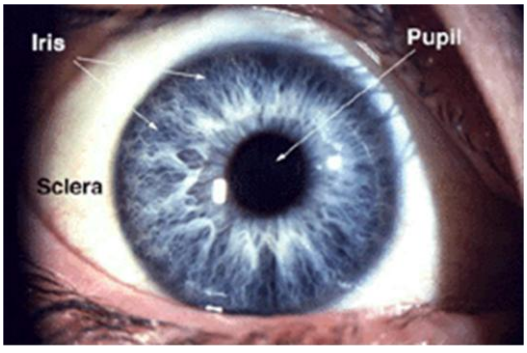

Figure 1: View of the human eye.

When looking at somebody's eyes, several structures are obvious. The **pupil** of the eye can be described as a dark cyclic structure in the central part, which is surrounded by the **iris**, a cyclic colored muscle which provides us the color of our eyes. Both pupil and iris are covered by a thin surface, the **cornea**, which allows the light rays to enter the eye. The cornea together with the **crystalline lens** forms a sharp image. The white part of the eye, the **sclera**, is continuous with the cornea and surrounds the iris and the pupil. the human eye,

three distinct layers are revealed: an external layer, which is formed by the **sclera** and the **cornea**, an intermediate layer that contains the **iris** and the **ciliary body** (anterior part), as well as the **choroid** (posterior part) and finally the internal layer or the **retina**, which is the sensory part of the eye. Furthermore, the human eye contains three chambers of fluid. The **anterior chamber**, located between the cornea and the iris, as well as the **posterior chamber**, between the iris and the crystalline lens, are filled with the **aqueous humor**, a water-like liquid that supplies the structures of these chambers with nutrients. On the other hand, the **vitreous chamber**, which is located between the back of the lens and the surface of the retina, is filled with the **vitreous humor**.

1.2 The anatomy of the retina

The **retina** is considered a part from the central nervous system (CNS), because it derives from the neural tube. During development, the retina is formed as an outpocket from the two sides of the neural tube, which is called optic vesicle. This optic vesicle undergoes further invagination to form the optic cup, with the inner part of the cup becoming the retina and the outer part giving rise to the **retinal pigment epithelium**, a melanin-containing structure located between the choroid and the retina, whose main function is the reduction of backscattering of the light that enters the retina, in order to produce a clear and sharp image (*Hilfer & Yang*, 1980).

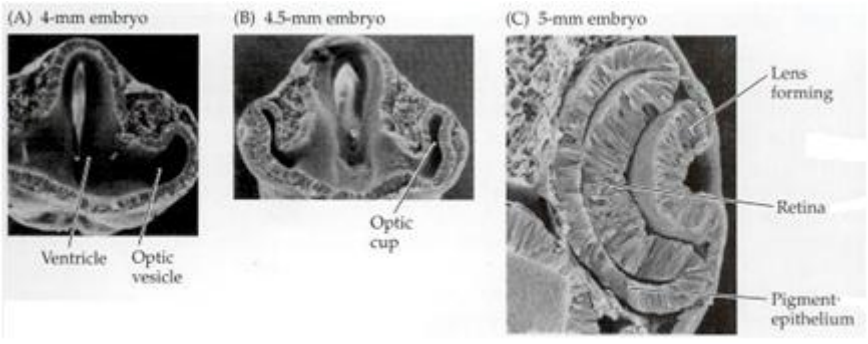

Figure 2: Development of the human eye. The retina is formed from the optic vesicle outpouching from the sides of the neural tube (A). The optic vesicle invaginates to form the optic cup from which the retina, the lens and the retinal pigment epithelium are formed (B,C). *Hilfer & Yang*, 1980.

Light rays are focused through the transparent cornea and the lens onto the retina. The central point where the image is focused in the human retina is called **fovea**. The field located approximately 6 mm around the fovea is considered as the **central retina**, which is surrounded by the **peripheral retina** (approximately 21 mm around the fovea). The retina is consisted by complex neural circuits that convert the electrical activity from the light-sensitive elements (the photoreceptors) to action potentials that are transmitted to the brain via the optic nerve axons *(webvision.med.utah.edu).*

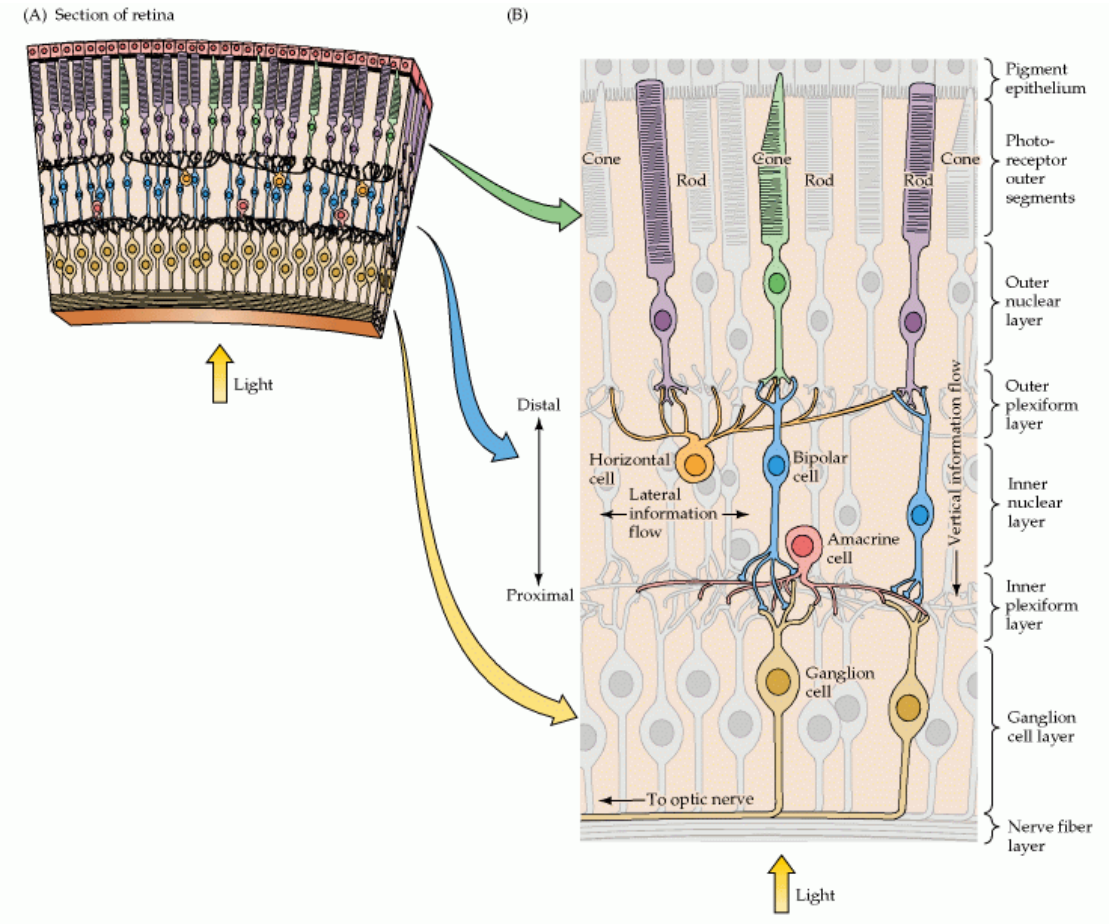

Figure 3: The organization of retinal neurons. (A) Section of the retina depicting the retinal layers. (B) Diagram of the neural circuitry of the retina showing the retinal cell types and their connections. (*Purves et al, 2001*)

The retina contains five major neurons, namely: **photoreceptors**, **bipolar**, **horizontal**, **amacrine** and **ganglion cells** which are organized in five layers, three layers of cells bodies and two layers of neuronal synapses (Fig. 3).

The cell bodies of the photoreceptors are located in the **outer nuclear layer** (ONL), while those of the bipolar, amacrine and horizontal cells lie in the **inner nuclear layer** (INL). The third layer of cell bodies is the **ganglion cell layer** (GCL) that contains the cell bodies of the ganglion cells, whose axons form the optic nerve, but also the bodies of displaced amacrine cells.

The photoreceptor terminals form synapses with the bipolar (vertically) and horizontal cells (horizontally) in the **outer plexiform layer** (OPL), while in the **inner plexiform layer** (IPL), the bipolar cells are connected to the ganglion cells. In addition, further interactions are observed between different types of amacrine cells, in order to amplify the ganglion cells' signal. The combination of this neural processing is required for the transmission of the visual image to the brain through the optic nerve *(webvision.med.utah.edu).*

As shown in Figure 3 the photoreceptors lie below the retinal pigment epithelium, while the ganglion cells are located to the inner part of the eyeball, therefore light passes the entire retinal layers in order to be captivated by the photoreceptors. The visual information is transmitted through the retina mainly via the vertical pathway consisting of the photoreceptors, rod bipolar cells and ganglion cells, whereas horizontal and amacrine cells are regulators of this pathway.

In addition, Müller cells, are the predominant type of glial cells found in the retina. These cells extend through the retinal layers from the outer nuclear to the ganglion cell layer (John E. Dowling, 1987). Other types of glial cells that have been observed in the retina are astrocytes (Ogden, 1978) and microglia (Boycott & Hopkins, 1981).

Retinal neurotransmitters

The major transmitter in the retina, released from the neurons comprising the vertical pathway, namely photoreceptors, bipolar and ganglion cells, is the excitatory amino acid glutamate. Horizontal cells release GABA, whereas there are about twenty different amacrine cells containing a variety of neurotransmitters/neuromodulators such as dopamine, acetylcholine, GABA, glycine, nitric oxide and various peptides. These neurotransmitters/neuromodulators mediate and play an important role in regulating the

physiology of the photoreceptors, rod bipolar and ganglion cells by interacting with their receptors found in the relative neurons.

1.3 Retinal diseases

Retinal diseases are characterized by one or more of the following components: neovascularization, neurodegeneration and inflammation. These mechanisms can induce retinal cell death, which leads to reduction of visual acuity and blindness. Retinal diseases, such as glaucoma, age related macular degeneration (ARMD) and diabetic retinopathy (DR) are considered important causes of low vision and blindness (Resnikoff et al., 2004). Glaucoma has recently been characterized as a neurodegenerative disease, as it is characterized by retinal ganglion cell loss (Harwerth & Quigley, 2006). However, the main characteristic of this disease is the high intraocular pressure (IOP) that can cause severe damage to the optic nerve. Treatment with antiglaucoma drugs may reduce the symptoms, namely reduce IOP, but does not stop the neurodegeneration of the ganglion cells.

ARMD, which is common in older people over 60 years of age is characterized by reduction of visual acuity in the center of the visual field. ARMD and diabetic retinopathy, observed in diabetes type I and II patients, are microvascular diseases characterized by neovascularization. In addition, retinal ischemia is believed to play a major role in the pathophysiology of these diseases. Extended retinal ischemia leads to bleeding, exudates and neovascularization. These pathologic features can result in retinal detachment, loss of visual acuity and blindness (Resnick et al., 2004). Recently neurodegeneration and inflammation have been suggested to be important components of DR (Antonetti et al., 2012). Many investigations have examined different targets as neuroprotectants of the retina by using different models of retinal disease.

Emphasis has been given to the protection of retinal ganglion cells, as they play a central role in the transmission of visual signals from the retina to the brain.However, other retinal cell types are also affected during the early stages of retinal degenerative diseases, such as horizontal and amacrine cells in diabetic retinopathy. The dysfunction of these retinal neurons can affect ganglion cells' function.

1.4 Retinal models of disease

Retinal ischemia

Ischemia is characterized by the lack of oxygen and glucose supply and the insufficient removal of waste products. As a result, normal neuronal membrane processes, as well as ionic homeostasis are altered, leading to presynaptic terminal membrane depolarization. Thus, depolarization results in an influx of calcium ions from the extracellular space into the presynaptic terminal, which triggers the release of glutamate from the presynaptic terminal into the synaptic cleft. The elevated levels of extracellular glutamate activate ionotropic glutamate receptors (NMDA and AMPA) found in the postsynaptic membrane (Vasilaki & Thermos, 2009, Hare et al., 2011). Consequently, voltage gated calcium channels are activated leading to an excess of intracellular calcium levels. Also, the formation of nitric oxide ($NO^{\cdot}$) is activated. These events are believed to mediate apoptosis and cell death (Osborne et al., 2004). This process is called ischemia-induced excitoxicity and it is considered the underlying cause of neurodegeneration in the retina that induces retinal cell loss. However, there is evidence that excitotoxic cell death can occur independently of extracellular and intracellular increases in calcium levels, but through extracellular high levels of chloride ions, which are observed after pathological activation of GABA or glycine receptors (Chen et al., 1998).

Glutamatergic excitotoxicity models

NMDA and AMPA models of excitotoxicity have been developed in order to study further the mechanisms of ischemia-induced neurotoxicity in CNS, including the retina, and discover new pharmacological targets that can provide neuroprotection to retinal neurons affected by the toxic insults (Andres et al., 2003, Hare et al., 2009, Kiagadaki & Thermos, 2008, Kokona & Thermos, 2015). Both NMDA and AMPA receptors are permeable to sodium and calcium ions, but NMDA-type channels have relatively higher calcium permeability. It is known that retinal ganglion cells express NMDA-type channels, thus excitotoxicity that is mediated by NMDA channels is believed to be an important mechanism for ganglion cell injury in several animal models of acute or chronic diseases, such as retinal ischemia and glaucoma (Hare et al., 2011). Intravitreal injections of NMDA have been shown to produce substantial damage in the inner nuclear, inner and outer

plexiform, as well as in ganglion cell layer of the adult rat retina (Sun et al., 2001) and primarily amacrine and ganglion cell loss (Siliprandi et al., 1992). On the other hand, intravitreal injections of AMPA into the adult rat eye have been reported to induce loss of cholinergic, nitric oxide synthase (bNOS)-expressing and GABA amacrine and calbindin-positive horizontal cells (Kiagiadaki and Thermos, 2008, Kokona et al., 2012), as well as astroglial and microglial reactions and calcium precipitation (Andres et al., 2003).

Excitotoxic effects of glutamate in these models may trigger a cascade of events leading to either apoptotic or necrotic neuronal cell damage. *In vitro* studies performed in isolated embryo chick retina, showed that a brief exposure to NMDA, activated caspase-3-like proteases and appeared apoptotic features, detected by the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) test, in which apoptotic nuclei can be recognized by the presence of strand breaks in the DNA. However, after a short exposure of the retinas to AMPA, none of the characteristics above were observed (Ientile et al., 2003). These findings are in agreement with Kokona & Thermos (2015) that reported that the cell death induced by AMPA excitotoxicity seems to be caspase-3-independent and may involve necrotic or necroptotic mechanisms.

1.5 The Endocannabinoid system

Thousands of years the marijuana plant *Cannabis Sativa* was widely used as a therapeutic in medicine. Back to ancient times it was used as a sedative or to treat inflammation and pain. Throughout the years, it was found that it exerts a lot of physiological and psychological effects. The active component of the plant has been isolated and identified as Δ^9-tetrahydrocannabinol (Δ^9-THC) (Gaoni & Mechoulam, 1964). THC binds to receptors distributed throughout the body to produce numerous beneficial effects. For example it has been referred to positively affect the symptoms of nausea, appetite stimulation, tumor growth and intraocular pressure (Mechoulam, 2002, Hollister, 1971, Iversen, 2003). Generally, there are a lot of studies that have proved that cannabinoids protect the CNS from a variety of pathologic conditions, such as neurotoxicity, trauma, oxidative stress and ischemia (Yazulla, 2008, Van der Stelt & Di Marzo, 2005).

Cannabinoid agonists and receptors

Δ^9-THC and a variety of synthetic cannabinoids mimic the effects of endogenous substances called endocannabinoids. Endocannabinoids are considered as one of the most recently identified neuromodulation system in the central nervous system. They were first studied in the 1990s. Firstly, the specific receptors that THC binds to, cannabinoid receptor type-1 (CB1) and cannabinoid receptor type-2 (CB2) were cloned and identified (Matsuda et al., 1990, Munro et al., 1993, Devane et al., 1988). These receptors belong to the G-protein coupled receptor superfamily and they are expressed in the CNS (CB1 receptor) or PNS (CB2 receptor) (Pertwee et al., 2006, 2010). The investigation of the cannabinoid receptors led to the identification of the endogenous ligands that they respond to (Devane et al., 1992, Mechoulam et al., 1995). There is evidence that proves the fact that endocannabinoids are not stored in "resting" cells, but they are synthesized and released in the CNS "on demand" evoked by pathological or physiological stimuli (Di Marzo et al., 1994).

Endocannabinoids belong to a large group of bioactive lipids which are called eicosanoids. All eicosanoids are derivatives of arachidonoid acid, a polyunsaturated fatty acid to which the endogenous cannabinoids are metabolized (Yazulla, 2008). The two most studied endocannabinoids are arachidonoylethanolamide (anandamide, AEA) and 2-arachidonoyl-glycerol (2-AG). Apart from these, there are several other endogenous substances that can bind as agonists to the cannabinoid receptors, such as O-arachidonoyl-ethanolamine (virhodamine), 2-arachidonylglyceryl ether (noladin ether), N-arachidonoyl-dopamine (NADA) and oleylethanolamine (OEA) (Pertwee et al., 2010, Howlett, 2002, De Petrocellis et al., 2004). The endocannabinoid 2-AG is the most abundant in the central nervous system compared to the others (Sugiura et al., 1995). All the cannabinoid agonists mentioned above have affinities for both or for one of the two cannabinoid receptors. For example 2-AG binds to both receptors as a full agonist, but it is slightly selective for binding to the CB1 over CB2 receptor (Howlett, 2002). On the other hand, anandamide behaves as a partial agonist at CB1 and CB2 receptors, while it exhibits higher CB1 than CB2 efficacy. Δ^9-THC mimics the behavior of anandamide in receptor binding (Pertwee, 1999). Apart from CB1 and CB2 receptors there are other non-cannabinoid receptors that can bind the endogenous compounds above. The most popular of these is the transient receptor potential of vanilloid type-1 (TRPV1) channel, which is the receptor for capsaicin, the purgent ingredient of hot chili peppers (Zygmunt et al., 1999, De Petrocellis & Di

Marzo, 2010). Anandamide but not 2-AG can bind and activate this channel receptor, but with lower potency and efficacy than to the CB1 receptor. Apart from TRPV1, the orphan receptors GPR55 (Ryberg et al., 2007) and GPR18 were found to be sensitive in cannabinoids, such as anandamide, 2-AG, PEA, virhodamine and noladin ether. These receptors are known to mediate the recruitment and activation of microglia during neuroinflammation and neuropathic pain (Gowran et al., 2011). Anandamide can activate the GRP55 receptor with a potency equivalent to that activating CB1 and CB2 receptors, while 2-AG, PEA and virhodamine show significantly more potent action through GPR55 than trough CB1 or CB2 receptors (Ryberg et al., 2007).

Apart from endogenous cannabinoids, plenty of synthetic compounds have been developed that bind to cannabinoid receptors and mimic the effects of the endocannabinoids or Δ^9-THC. Of these, the most studied are R-(+)-methanandamide (synthetic analogue of anadamide), which is CB1-selective (Pertwee & Ross, 2002), HU-210 (synthetic analogue of (Δ^9-THC) that displays high affinity for both CB1 and CB2 receptors, R-(+)-WIN55,212-2 and CP55,940, which have affinities for both receptors but lower than those of HU-210 (Pertwee et al 2010). From all these analogues, HU-210, which was first described by Mechoulam et al 1988, is the most potent and it displays the highest relative intrinsic activity as a cannabinoid agonist. In fact, it has been shown that HU-210 is seven times more potent than Δ^9-THC at binding to CB1 receptor (De Vry et al 2004, Howlett et al 1990).

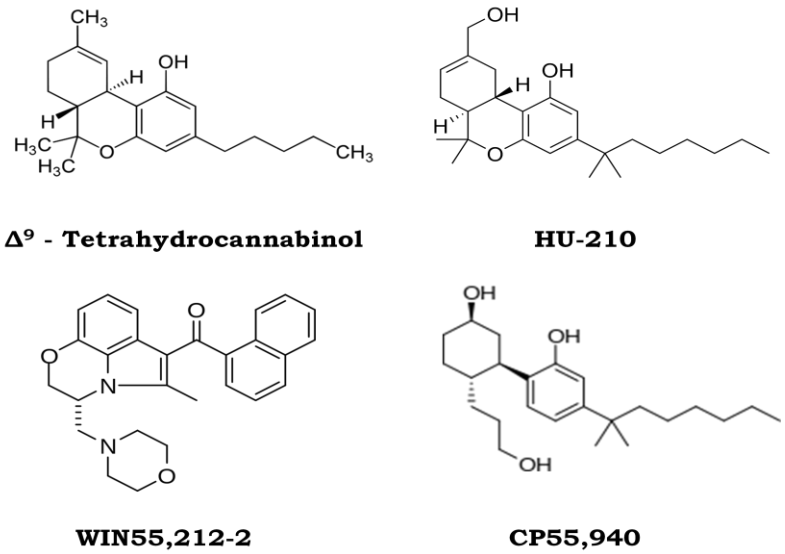

Figure 4: Structures of Δ^9-Tetrahydrocannabinol and synthetic analogues HU-210, WIN55,212-2 and CP55,940

Cannabinoid metabolic pathways

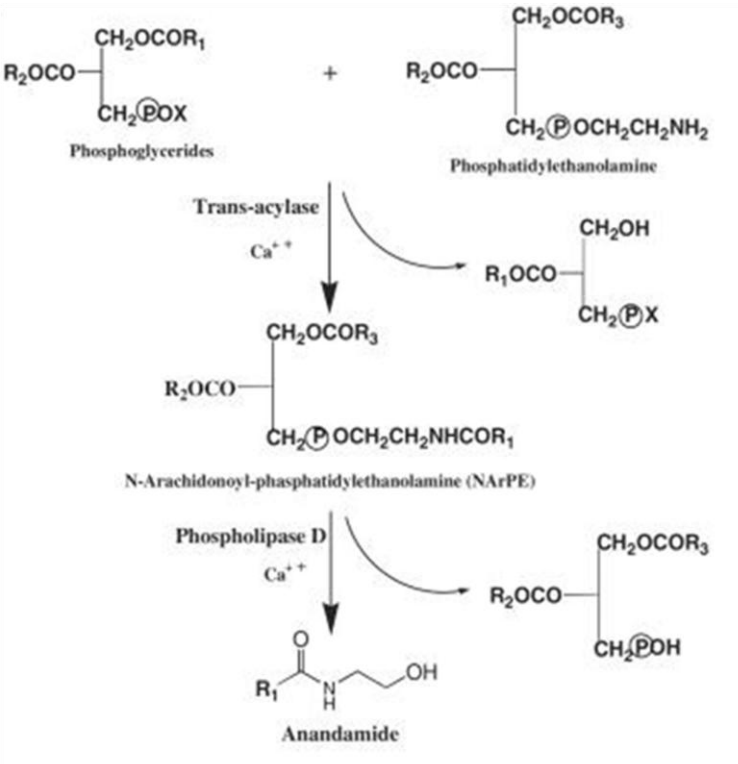

Figure 5: Potent pathway for anandamide's biosynthesis. (De Petrocellis et al 2004)

The biosynthesis of the two most important endocannabinoids, anandamide and 2-AG follows different pathways. Firstly, anandamide which belongs to a class of lipids named N-acyl-phosphatidylethanolamines (NAPEs), is synthesized via a phospholipid-depended pathway. The main enzyme that catalyzes the reactions is a phospholipase D, selective for NAPEs (NAPE-PLD). Anandamide's precursor molecule is N-arachidonoyl-phosphatidylethanolamine (NArPE) whose enzymatic hydrolysis produces anandamide and phosphatidic acid (Fig.5) (De Petrocellis et al 2004).

On the other hand, several pathways have been proposed for the biosynthesis of 2-AG. In most cases it is produced from the hydrolysis of its precursor molecules 2-arachidonate-containing diacylglycerols (DAGs) by the enzyme DAG lipase (DAGL), which catalyzes the transformation of diacylglycerols into monoacylglycerols (Sugiura et al 2002, Bisogno et al 2003). More specifically, diacylglycerol (DAG) and inositol triphosphate (IP3) are produced, via the action of phospholipase C (PLC), from phosphatidylinositol 4,5-biphosphate (PIP2). Then, DAG is transformed into 2-AG by DAGL. So far, two isoforms of this enzyme have been found in across the plasma membrane and according to the literature, only the isoform DAGLα is expressed in the brain (Bisogno et al 2003, Katona et al 2006).

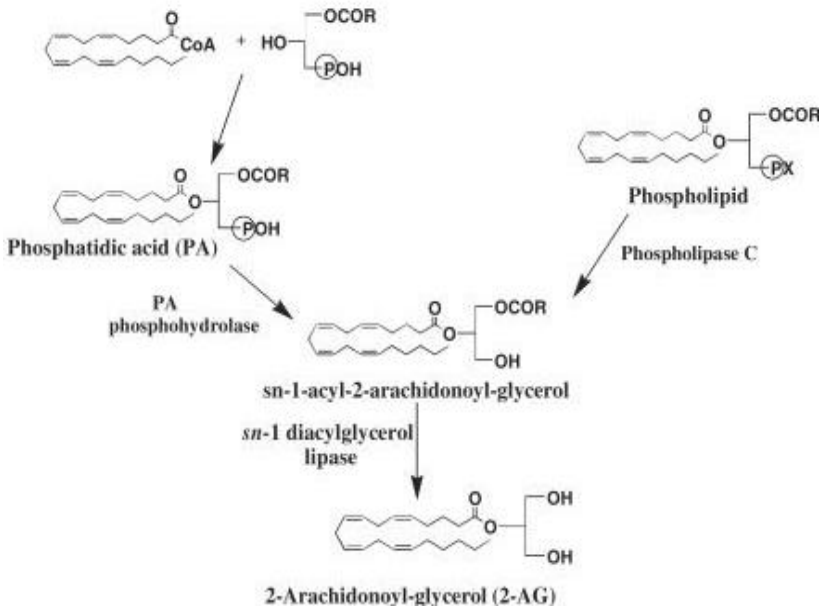

Figure 6: Potent pathway for 2-arachidonoyl-glycerol's biosynthesis. (De Petrocellis et al., 2004)

Cannabinoids are lipofilic, so they can diffuse through the cell membrane to balance their concentration between the extracellular and intracellular space. In order to be inactivated rapidly and removed from their extracellular targets, the two basic cannabinoids anandamide and 2-AG, can be taken up from the synaptic cleft by neurons or glial cells by a selective mechanism, named anandamide membrane transporter (AMT) (Beltramo et al., 1997, Bisogno et al., 1997, Maccarone et al., 1998). After their removal from their sides of action by this cellular uptake system, they are metabolized by two major enzymes, fatty acid amide hydrolase -1 (FAAH-1) and monoacylglycerol lipase (MAGL) (Fig.7). FAAH is the main enzyme for anandamide's degradation to arachidonic acid and ethanolamine, while 2-AG is metabolized by MAGL to arachidonic acid and glycerol (Pertwee 2006, De Petrocellis et al., 2004, Dinh et al., 2002). Furthermore, there is evidence that anandamide can be catabolized by the enzyme FAAH-2, to a much lesser extent, which is not expressed in rodents and there are other two enzymes that can hydrolyze 2-AG, α,β-hydrolase-6 (ABHD6) and α,β-hydrolase-12 (ABHD12). Also even FAAH-1 takes part in the catabolic procedure of 2-AG, to a lesser extent (Feledziak et al., 2012, Savinaien et al., 2012). Apart from hydrolysis, endocannabinoids can also undergo enzymatic oxidation of their arachidonic moiety by enzymes such as cyclooxygenase-2 (COX-2), lipooxygenases (LOX) and cytochrome p450 oxidases. Enzymatic hydrolysis by COX-2 of anandamide and 2-AG produces biologically active prostaglandins (Kozak & Marnett, 2002).

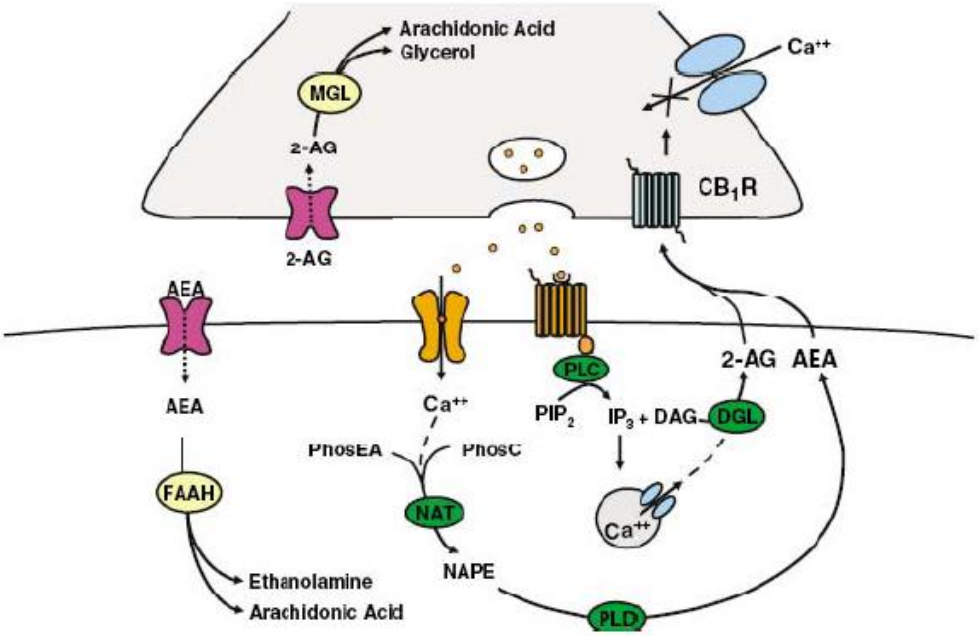

Figure 7: Schematic representation of possible biosynthetic and catabolic pathways of endocannabinoids within a synapse (Rea et al., 2007).

Cannabinoid signaling

The two cannabinoid receptors, CB1 and CB2, are 7-transmembrane region receptors and their activation can trigger several transduction pathways involved in cell survival, proliferation and differentiation. They couple to both Gi/o and Gs proteins in order to modulate cAMP levels, through the inhibition or activation of adenylyl cyclase (AC), respectively (Bonhaus et al., 1998), where activation and inhibition depend on the different isoforms of AC. The inhibition of AC by the activation of cannabinoid receptors is one of the most common cannabinoid actions (Howlett and Flemming, 1984), and it has been studied in both neurons that express the CB1 receptor and lymphocytes that express the CB2 receptors.

Furthermore, the activation of these receptors can regulate specific ion channels. For example, the activation of the CB1 receptor by endogenous or synthetic cannabinoids (such as anandamide, 2-AG, WIN55,212-2 and CP55940) can lead to the inhibition of L,N,Q and P-type Ca^{2+} channels (Mackie and Hille, 1992 Mackie et al., 1995). This inhibition is cAMP independent (Mackie et al., 1993). Also, the activation of the CB1

receptor subsequently activates inward rectifying K^+ channels (Demuth & Molleman, 2006, Mackie et al., 1995).

Cannabinoids also act through the regulation of certain intracellular kinases. The activation of both CB1 and CB2 receptors can activate mitogen-activated protein kinases (MAPKs) and phosphoinositide 3-kinase (PI3K). MAPK downstream signaling, after cannabinoid activation, can lead to the further activation of ERK1/2, p38-MAPK and JNK kinases (Bouaboula et al., 1996, Wartmann et al., 1995), while PI3K phpsphorylates and thus activates protein kinase B (PKB or Akt) (Gomez del Pulgar et al., 2000, Sanchez et al., 2003). CB1 receptors can also activate phopsholipase C (PLC) signaling pathways (Bouaboula et al., 1995), while CB2 receptors, on the other hand, are activators of ceramide biosynthesis (Howlett et al., 2004).

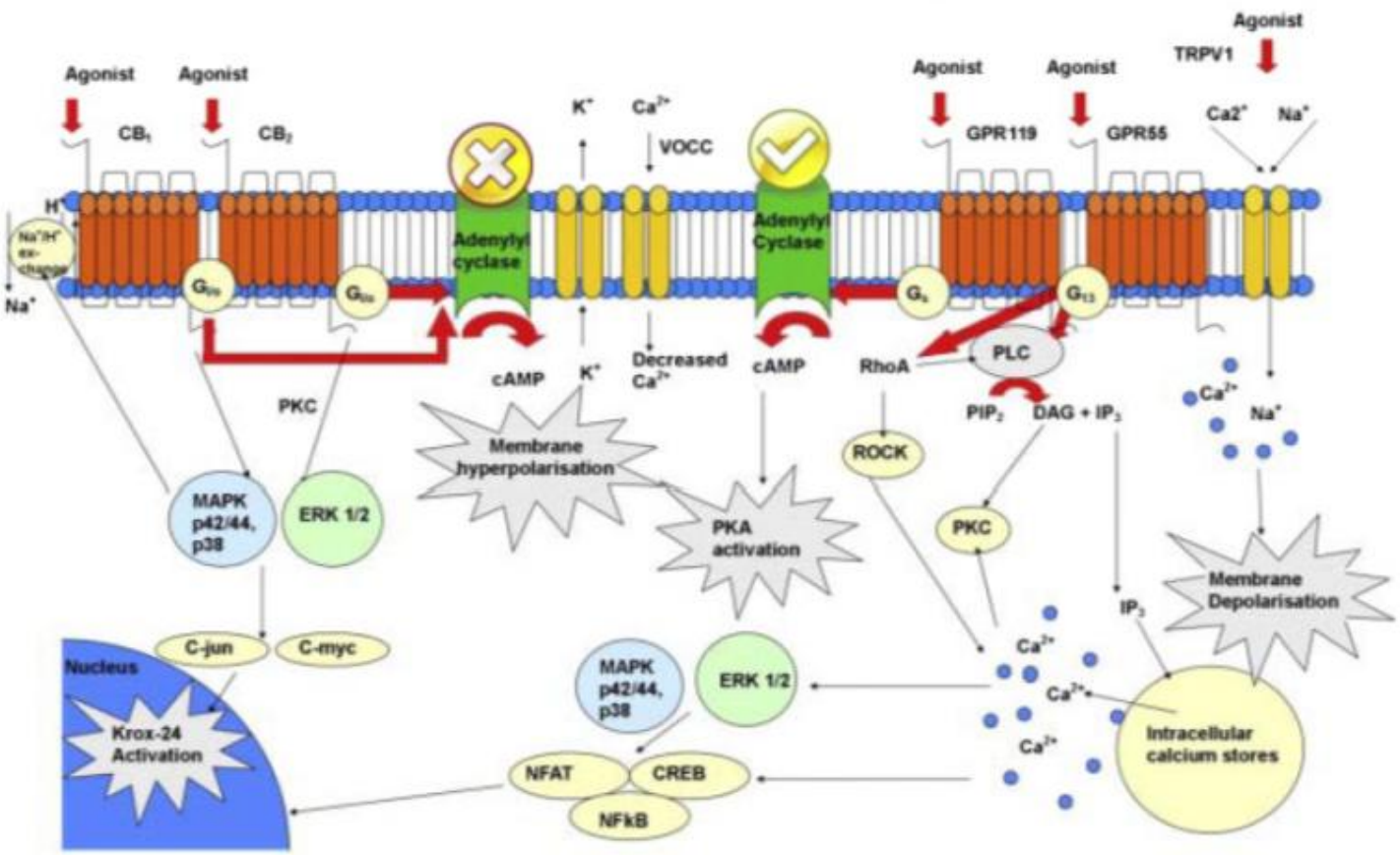

Figure 8: Cannabinoid receptor signaling cascades (Ladak et al., 2011).

Endocannabinoids, especially anandamide and 2-AG, have different functions, depending on the receptor that they act on (Fig.9). They are thought to be produced presynaptically or postsynaptically "on demand" (anandamide and 2-AG respectively) and it is thought that both of them act as neurotransmitters and neuromodulators (Di Marzo et al., 1998, 1999), but also as retrograde synaptic messengers (Pertwee & Ross, 2002), as they can act through presynaptic CB1 receptors and inhibit the presynaptic release of GABA or glutamate in specific brain areas (Wilson et al., 2001, Kreitzer et al., 2001). Anandamide can act through post-synaptic TRPV1 receptors and decrease glutamate release and induce long-term depression (LTD) by stimulating AMPAR endocytosis, or through pre-synaptic TRPV1 receptors to increase it (Grueter et al., 2010). On the other hand, 2-AG can bind to CB2 receptors in astrocytes and microglia in order to reduce inflammation (Stella, 2010). This function implies that this cannabinoid can be a potential target for the treatment of neuroinflammatory diseases. Also, it can act through CB1 receptors as well in astrocytes to mediate several biological functions depicted in Fig. 9 (Navarrete et al., 2010, Han et al., 2012, Min & Nevian, 2012).

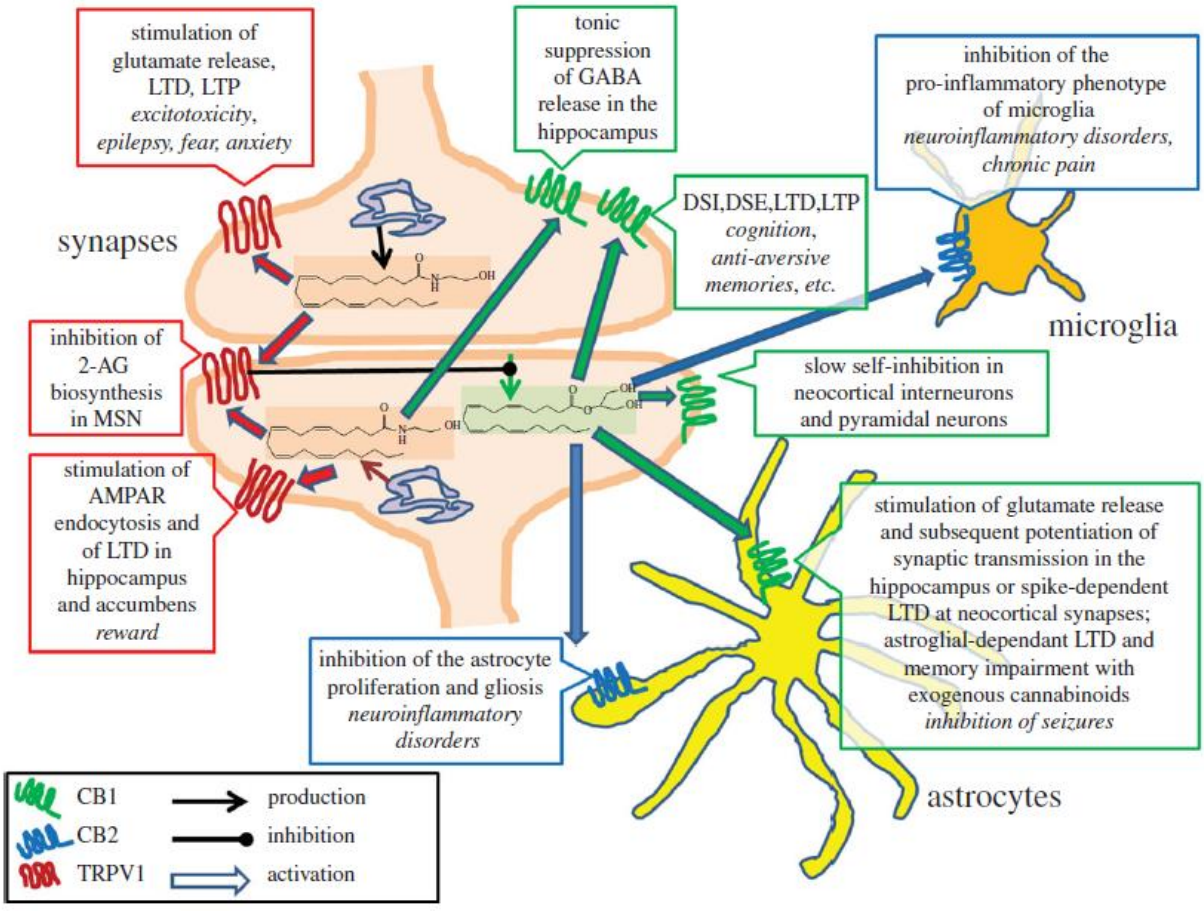

Figure 9: Different functions of cannabinoids in neurons and glia. (Di Marzo & De Petrocellis, 2012).

Endocannabinoids in the retina

The retina, as a part of the CNS, comprises a functional endocannabinoid system, which includes the endogenous cannabinoid agonists and their receptors and as well as the enzymes that are responsible for their synthesis, transport and degradation.

There are plenty of studies in the literature that have investigated the presence of the endocannabinoids in the retina (Bisogno et al., 1999, Chen et al., 2005, Straiker et al., 1999). It seems that 2-AG is much more abundant than anandamide in the vertebrate retina. The mRNA of the CB1 receptor was first detected in the retina of the embryonic rat by in situ hybridization (Buckley et al., 1998). Since then the mRNA and the CB1R protein as well, were found to be expressed in high levels in several subregions of the human eye, including the retina, as detected by in situ-hybridization and western blot analysis, respectively (Porcella et al., 2000). Also, there are numerous studies that support the presence and function of CB1 receptor in the vertebrate retina. Straiker et al. (1999) reported that the CB1 receptor was found in high concentration in the two synaptic retinal layers, the outer and inner plexiform layer (OPL and IPL), in rat, mouse, rhesus monkey, chick, goldfish and tiger salamander retina by immunohistochemical studies. In all the species examined, except from salamander, a robust staining of the OPL was observed, suggesting the presence of the CB1 receptor in cone pedicles and rod spherules, the synaptic terminals of cone and rod photoreceptors. The CB1R immunoreactivity in the IPL was detected in all the species examined. The expression of CB1 receptor in photoreceptor terminals is also confirmed by Yazulla et al. (1999) and Hu et al. (2010). In the last study, CB1R immunoreactivity was found to be colocalized with FAAH immunoreactivity in the OPL, in subpopulations of amacrine cells in INL and in cells in the GCL in mouse retina. Furthermore, other studies have reported the expression of the CB1R in rod bipolar, amacrine and horizontal cells in rat retina (Yazulla et al., 1999), in the ganglion cell nucleus and axons in monkey retina (Bouskila et al., 2012), as well as in horizontal, amacrine cells and ganglion cells in rat retina (Zabouri et al., 2011). Finally, CB1 receptor was not found to be expressed in Muller cells in retina (Bouskila et al., 2012). All this information is summarized in the table below.

Cell type or retinal layer	Species	Reference
Outer nuclear layer (ONL)	Rat	Yazulla et al 1999
Outer plexiform layer (OPL)	Mouse, rat, monkey, goldfish, chick, salamander	Straiker et al 1999, Yazulla et al 1999, Shu-Jung Hu et al 2010
Inner nuclear layer (INL)	Mouse, Rat	Bouskila et al 2012, Yazulla et al 1999
Inner plexiform layer (IPL)	Mouse, rat, monkey, goldfish, chick	Straiker et al 1999, Yazulla et al 1999
Ganglion cell layer (GCL)	Mouse	Bouskila et al 2012
Photoreceptors	Monkey	Bouskila et al 2012
Rod bipolar cells	Monkey, rat	Bouskila et al 2012, Yazulla et al 1999
Horizontal cells	Rat	Yazulla et al 1999
Amacrine cells, GABA amacrine cells	Mouse, rat	Shu-Jung Hu et al 2010, Zabouri et al 2011, Yazulla et al 1999
Ganglion cells and axons	Mouse, rat, monkey	Shu-Jung Hu et al 2010, Zabouri et al 2011, Bouskila et al 2012

Table 1: Expression of CB1 receptor in retinal neurons and layers in different species.

The presence of the CB2 receptor in the retina has been studied for many years, as it is not clear yet if it is expressed in the CNS in general. Nevertheless, there are several studies that report the expression of this receptor in the retina. First of all, the mRNA of the CB2 receptor has been detected by in situ hybridization within the adult rat retina, with strong signal in the inner segments of photoreceptors, ONL, INL and GCL (Lu et al., 2000). Moreover, immunohistochemical studies by Lopez et al. (2011) revealed the expression of the CB2 receptor in amacrine, horizontal and ganglion cells in adult rat retina and Bouskila et al. (2013), reported that the CB2 receptor is localized in Muller cells in the vervet monkey retina. Postsynaptic localization of these receptors is more likely (Gong et al., 2006, Onaivi et al., 2006). Furthermore, a recent study shown that the CB2 receptor is widely distributed in adult mouse retina, where its immunoreactivity was found in inner outer segments and cell bodies of both cone and rod photoreceptors and therefore in rod and cone bipolar cells, as well as in some amacrine and ganglion cells somata (Cecyre et al., 2013). However, the same group later published one study where several available antibodies that target the CB2 receptor was tested by immunohistochemistry and western

blot analysis for their specificity. The authors concluded that the use of those antibodies could not provide reliable information, due to their low specificity (Cecyre et al., 2014).

The metabolic enzyme FAAH that is responsible for the degradation of anandamide is widely expressed in mouse retina and more specifically in inner segments of photoreceptors, ONL, GCL and in the axon terminals of photoreceptors in the OPL. Also, it has been shown that it is co-expressed with the CB1 receptor in some amacrine cells and ganglion cells (Shu-Jung Hu et al., 2010). Moreover, Yazulla et al., 1999 reported that FAAH is expressed in horizontal and PKC-positive bipolar and amacrine cells, as detected by double-label immunohistochemistry in rat retina. On the other hand, MAGL, the enzyme that catabolizes 2-AG, was found to be present in cone pedicles and rod spherules in the OPL, as well as in two laminae of the IPL of mouse retina (Shu-Jung Hu et al., 2010).

1.6 Neuroprotective effects of cannabinoids in the retina

The significance of cannabinoids in ocular physiology has already been known for many years, since Hepler and Frank reported in 1971, that subjects who were smoking marijuana developed lower intraocular pressure (IOP). To date, cannabinoids have been reported to provide neuroprotection to retinal neurons in animal models of retinopathies. To start with, the psychotropic Δ^9-THC has been shown to protect retinal ganglion cells against NMDA excitotoxicity through the activation of the CB1 receptor. On the other hand, the natural and non-psuchotropic compound cannabidiol (CBD) protected ganglion cells as well, but the mechanism of this action is unknown, as this compound does not activate CB1 receptors (El-Remessy et al., 2003). Additionally, the administration of the synthetic cannabiboid methanandamide, rescued retinal ganglion cells in an *in vivo* model of ischemia reperfussion, via the activation of the CB1 and TRPV1 receptors (Nucci et al., 2007). More recently, a study showed that the synthetic HU-210 provided neuroprotection against photoreceptor degeneration in an *in vivo* model of retinitis pigmentosa (Lax et al., 2014). Also, several studies are available that reported the ability of cannabinoids, such as 2-AG, dexanabinol (HU-211) and WIN55,212-2, to lower intraocular pressure, which implicates that cannabinoids can be potentially used in the treatment of glaucoma (Laine et al., 2002, Beilin et al., 2000, Song & Slowey, 2000). According to Kokona & Thermos,

2015, the endogenous compound anandamide, but also, the synthetic cannabinoids HU-210 and methanandamide, can protect retinal neurons against AMPA excitotoxicity. The excitatory amino acid AMPA has been shown to induce cholinergic, nitric oxide synthase (bNOS)-expressing and GABA amacrine, as well as calbindin-immunoreactive horizontal cell loss (Kiagiadaki and Thermos, 2008, Kokona et al., 2012). In this study, the cannabinoids mentioned above, when administered exogenously prevented cholinergic and bNOS-positive amacrine and horizontal cell loss, via the activation of the CB1 receptor. Also, PI3K/Akt and/or ERK1/2 signaling prosurvival pathways were shown to be involved in the mechanism of neuroprotection provided by these compounds.

Moreover, inhibition of the metabolic enzymes of the endogenous cannabinoids, FAAH and MAGL, was found to afford neuroprotection as well to the same retinal neurons as mentioned above (Kokona et al., unpublished data). However, the co-administration of the inhibitors of the metabolic enzymes with anandamide, 2-AG and HU-210 exogenously, failed to prevent retinal cell loss that was induced by AMPA excitotoxicity. A possible explanation for this observation is that the elevation of the endogenous cannabinoid levels in combination with the exogenous administered cannabinoids, induced down-regulation of the CB1 receptor. As a consequence, the neuroprotective actions of the cannabinoids were not evident.

In the literature, there is evidence that the prolonged exposure of CB1 receptor to agonists to brain areas, leads to desensitization and downregulation of the receptor. Repeated administration of Δ^9-THC or WIN55,212-5 induces profound tolerance that correlated with desensitization and downregulation of CB1 cannabinoid receptors in brain regions, such as the hippocampus, substantia nigra and globus pallidus (Sim-Seley et al., 2002, 2006). . Prolonged exposure to WIN55,212-2 was found to desensitize the cannabinoid-induced presynaptic inhibition of glutamatergic neurotransmission in rat hippocampal neurons *in vitro* (Kouznetsova et al., 2002). Apart from THC and WIN55,212-2, the synthetic cannabinoid HU-210 has also been referred to induce down-regulation of the CB1 receptor in several brain areas, as its chronic, subchronic and acute administration, in different doses, has been found to decrease CB1 receptor binding in adolescent and adult rat brain, suggesting that this receptor adaptation appears to be dose-dependent and region-specific and leads to the development of cannabinoid tolerance (Dalton et al., 2009, 2010).

In Figure 10, a very common pathway that might be involved in the mechanism through which the CB1 receptor can be down-regulated is shown, namely the GRK/arrestin pathway (Smith et al., 2010). This is a general mechanism that takes place when a G-protein coupled receptor is chronically activated by agonists. In this pathway, an activated GPCR is phosphorylated on C-terminal Ser/Thr residues, by one of the several GRKs. Once phosphorylated, β-arrestin can bind to the GPCR desensitizing the receptor and causing it to internalize via clathrin-coated pits. Once internalized, GPCRs may be recycled back to the cell surface following dephosphorylation in acidified endosomal compartments. Alternatively, GPCRs can be trafficked to lysosomes and degraded, a process that is facilitated by GPCR-associated sorting protein (GASP1).

According to the literature, chronic Δ^9-THC treatment enhanced the expression of GRK2 and 4 and b-arrestin-1 and 2 in mouse brain regions (Rubino et al., 2006). In addition, desensitization of CB1-mediated inhibition of glutamatergic neurotransmission in hippocampal neurons was blocked by the expression of dominant negative mutants of GRK2 or b-arrestin2 (Kouznetsova et al., 2002). It has not been proved yet if the mechanism shown in Figure 10 is involved in the down-regulation of the CB1 receptor, as there is little evidence of CB1 direct interaction with GRKs and β-arrestin. There is one study that showed the interaction of a synthetic peptide corresponding to residues 419–439 of the CB1 receptor with b-arrestin-2 (Bakshi et al., 2007), and it has been also shown that CB1 receptors are co-localized with GASP1 in rat hippocampal, spinal cord and striatal neurons (Tappe-Theodor et al., 2007).

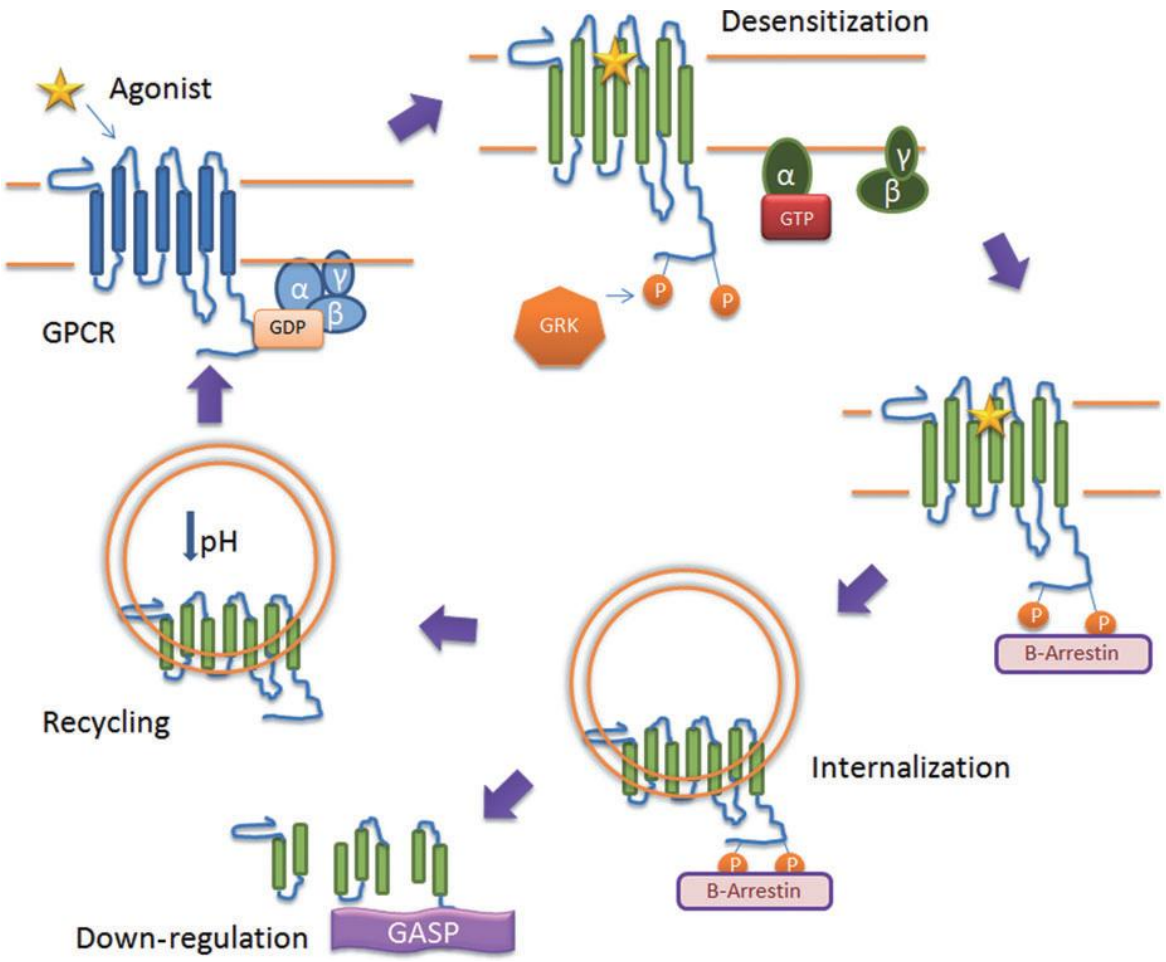

Figure 10: The GRK/arrestin pathway: a mechanism for G-protein-coupled receptor (GPCR) desensitization, internalization and down-regulation (Smith et al., 2010).

Considering the information mentioned above, the discovery of new pharmacological targets that will provide neuroprotection and increase the viability of retinal neurons and visual acuity is essential. Cannabinoids, due to their neuroprotective role, could be proposed as candidates for thearapeutics in the threatment of retinal diseases. However, further investigation is necessary for the elucidation of the role of the endocannabinoid system in healthy retina and in retinal ischemic conditions.

2. Aim of study

Endogenous and synthetic cannabinoids have been reported to afford neuroprotection to the retina in different models of neurotoxicity (Kokona et al., 2016). Recently it was shown that the neuroprotective effects of the endocannabinoid anandamide and the synthetic HU-210, are mediated via CB1 receptor activation and the PI3K/Akt and ERK1/2 signaling pathways have been shown to be involved in the mechanism of neuroprotection (Kokona & Thermos, 2015). However, HU-210 has been shown to induce downregulation in CB1 receptor binding in several brain areas in rat, after chronic treatment (Dalton et al., 2009). To date, there is no evidence to suggest that CB1 receptor downregulation occurs in the retina. Therefore, information about the chronic effects of cannabinoids in the retina would be very important in order to recommend these agents as potential therapeutics in retinopathies.

This study focuses on A) the investigation of the possible downregulation of the CB1 receptor after chronic or subchronic administration of HU-210 in healthy rat retinas and its effect on the downstream signaling pathway PI3K/Akt, involved in the actions of HU210 and B) the investigation of the downregulation of the CB1 receptor using the same conditions of HU-210 administration in ischemic rat retina (*in vivo* model of AMPA excitotoxicity). The results from this study will provide information regarding the efficacy of HU-210 as a neuroprotectant in chronic retinal disease.

3. Materials & Methods

3.1 Animals

Adult male and female Sprague-Dawley (250-300g) rats were employed in all studies in accordance with the EU Directive 2010/63/EU, the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and in compliance with Greek national laws (EC Directive 1986/609 and Law 2015/2001). The animals were housed 2 to 3 according to their sex and maintained on a 12 h light/dark cycle with food and water ad libitum. Room temperature was maintained at 22°C. Euthanization was performed with diethyl ether inhalation. All protocols were approved by local Veterinarian Authorities.

3.2 Drugs & treatments

The synthetic cannabinoid HU-210 (Tocris, Bristol, UK) was dissolved in a vehicle solution of absolute ethanol/water for injection (EtOH/WFI) and administered by intraperitoneal (i.p.) injections for different times.

Chronic, subchronic and acute treatment

Adult Sprague-Dawley rats were injected (i.p.) with HU-210 daily for 14 days (chronic treatment), and for 4 days (subchronic treatment). In order to study the acute effects, the rats were injected 13 days with vehicle and then received a single dose of HU-210 on the 14th day (Dalton et al 2009).

Dose-dependency studies: The animals were divided in two groups, the control and the HU210-treatment group. The rats were injected intraperitoneally (i.p.) with HU-210 (25, 50 or 100 μg/kg in vehicle). Control rats were in injected with the vehicle EtOH/WFI (2.5, 5, 10 μl/ml respectively).

Twenty four hours after the last intraperitoneal injection, rats were euthanatized and the eyes were removed to collect eyecups or retinas for immunohistochemical or western blot studies, respectively.

The experimental animal groups are summarized in the table below:

Experimental group	Treatment (i.p.)	Duration of treatment
Control	Vehicle	Acute, 4 days, 14 days
HU-210	25, 50, 100 μg/kg in EtOH/WFI	Acute, 4 days, 14 days

3.3 AMPA-induced excitotoxicity model & treatment

The in vivo model of AMPA excitotoxicity was employed according to Kiagiadaki & Thermos, 2008. The animals were anesthetized with intramuscular injections of xylazine (15.5 mg/kg) and ketamine (100 mg/kg) and placed in a stereotaxic apparatus to stabilize the head and facilitate intravitreal administration. Intravitreal injections were performed with 27-gauge needles connected to a Hamilton syringe adapted to a minipump, with a flow rate of 1μL/min for 5 minutes. The tip of the needle was inserted behind the sclera–cornea border into the vitreous humor. Rat eyes received intravitreally 5 μl PBS (50 mM K2HPO4/NaH2PO4, 0.9% NaCl, pH 7.4: Control) or 5 μl AMPA (42 nmol per eye, diluted in 50 mM PBS; Tocris, Bristol UK) or 5 μl AMPA with HU-210 (10^{-6} M ;Tocris, Bristol, UK), according to Kokona & Thermos, 2015.

Twenty four hours later after the intravitreal injections, the rats were injected intraperitoneally with HU-210, daily for 14 days (chronic treatment) or for 4 days (subchronic treatment) with the dose of 50 μg/kg (i.p), that was chosen from the previous studies in control animals. In order to study the acute effects of the treatment in this model, HU-210 (10^{-6}M) was co-administered with AMPA (8.4 mM) intravitreally, according to Kokona & Thermos (2015).

The treatments of the experimental animal groups in the model of AMPA excitotoxicity are summarized in the table below:

	Experimental groups			
Route of administration	**Control**	**AMPA**	**HU-210**	**AMPA & HU-210**
Intravitreally	PBS 50mM	AMPA 8.4 mM	AMPA 8.4 mM	AMPA 8.4 mM & HU-210 (10^{-6} M)
Intraperitoneally	Vehicle	Vehicle	HU-210 (50 μg/kg)	-

Twenty four hours after the last intraperitoneal injection, rats were euthanatized and the eyes were removed to collect eyecups or retinas for immunohistochemical studies or western blot analysis, respectively. In the acute experiment, rats were sacrificed 24h after the single intravitreal injection.

3.4 Immunohistochemistry

Tissue preparation

In order to isolate the eyecups (posterior part of the eye) the eyes were first fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer for 45 min at 4°C. Then, the anterior part of the eyeballs (cornea, lens and vitreous humor) was removed and the eyecups were further fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 1.5 h at 4°C. When isolated, the eyecups were rinsed in phosphate buffer and incubated in 30% sucrose overnight at 4 °C for cryoprotection. Tissues were frozen in isopentane at -45°C for 1 minute, using O.C.T compound (optimal cutting temperature compound) and kept at -80°C until further use. Eyecups were sectioned vertically near the optic nerve head using a cryostat (Superfrost; Erie Scientific, Portsmouth, NH) at -25°C and stored at -20°C. Serial vertical sections of 10 μm each were spread into 4 gelatin covered slides (6 sections per slide), so that each slide contained a representative part of the central retina, including the optic nerve head.

Immunohistochemical studies

Immunohistochemical staining was performed in order to study the areas or the specific retinal cell types that were affected by the synthetic cannabinoid HU-210 or the excitatory amino acid AMPA. Cryostat sections were washed twice with 0.1M Tris-HCl buffer; TBS, pH 7.4 (10 min each wash). Then the sections were incubated with blocking buffer; 0.1M

TBS, containing 3.3% normal goat serum for 30min, to block the non-specific binding sites of the sections, washed three times in 0.1M TBS (5 min each wash) and subsequently incubated with the primary antibodies, anti-cannabinoid receptor type-1 (CB1R, rabbit polyclonal IgG, 1:300, Abcam) or brain nitric oxide synthase (bNOS, rabbit polyclonal, 1:2000, Sigma) in 0.1M TBS containing 0.3% Triton X-100 and 0.5% normal goat serum overnight at room temperature. The sections were washed three times with 0.1M TBS (5 min each) and incubated for 1.5 hour with the secondary antibody, CF543 goat anti-rabbit IgG (H+L) (1:1000,Biotium). Finally, the sections were washed as previously and cover slipped with mounting medium with DAPI. Negative controls were obtained following the same protocol and by omitting the primary antibody.

3.3.3 Colocalization studies

Colocalization studies were performed with the anti-CB1R (rabbit polyclonal IgG, 1:300, Abcam) and anti-β-tubulin (neuronal class III, mouse monoclonal IgG2a, 1:2000, Covance) antibodies. Sections were incubated with the primary anti-CB1R antibody following the procedure above, and after the incubation with the anti-rabbit secondary antibody, they were washed and blocked again with normal goat serum. Then they were incubated overnight with the second primary anti-β-tubulin antibody, as mentioned above. Finally, the sections were incubated with the second secondary antibody, CF488A goat anti-mouse IgG (H+L) (1:400,Biotium) and cover slipped with mounting medium with DAPI.

Antibody	Host	Working dilution	Company	Localization of IR in retina
bNOS	Rabbit	1:2000	Sigma	Amacrine cells
CB1R	Rabbit	1:200	Abcam	OPL, INL, IPL, GCL
β-tubulin III	Mouse	1:2000	Covance	IPL, ganglion cells

*Abbreviations: IR = immunoreactivity

3.5 Microscopy

Light microscopy images were taken with a camera (Leica DC 300F 40/0.70; Leica DMLB). Light and contrast adjustments of images were processed with the use of Photoshop ver. 7.0 software (Adobe Systems, San Jose, CA).

3.6 Quantification studies

In order to quantify the CB1R immunoreactivity (CB1R-IR), two photographs were taken from 3 slices (2 photos/ slice) of each retina near the optic nerve head (central retina), containing the outer plexiform (OPL), inner nuclear (INL), inner plexiform (IPL) and ganglion cell (GCL) layers. The GCL or the area containing the INL, IPL and GCL were delineated, for quantification of the control retinal sections and sections from the AMPA excitotoxicity model respectively, using the ImageJ 1.44 software and the mean grey value for the CB1R-IR of this region was calculated in each image. Finally the mean of the six values was used for each retina.

Additionally, bNOS-IR positive retinal neurons were counted along the entire tissue, in the INL (bNOS expressing amacrine cells) and GCL (displaced amacrine cells). bNOS-IR possitive cells were counted in 3 slices of each retina.

Quantification studies were carried out at least for 3 retinas in each treatment, for both CB1R and bNOS immunoreactivity.

3.7 Statistical analysis

Quantification data was expressed as percentage of control (100%) and analyzed using GraphPad Prism 5.0 (GraphPad Software, Inc, San Diego, CA, USA) one-way ANOVA with Newman-Keuls post hoc analysis, two-way ANOVA with Bonferoni post hoc analysis, or two-tailed t-test. Differences between the groups was considered statistically significant when $p < 0.05$. Finally, data were plotted as the mean ± S.E.M (Standard Error of the Mean) of all values in the different groups.

3.8 Western blot analysis

Sample preparation

Retinas were homogenized mechanically and sonicated in lysis buffer, containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP40, 0.1% DOC, 0.1 mM PMSF and a protease and phosphatase inhibitor cocktail (Sigma,Thermo Sci., respectively) using the following protocol: 500μl of lysis buffer were added to each retina. The samples were centrifuged at 10.000g for 20 minutes, at 4°C. Then, the supernatant was collected and split in two, so that in each 250 μl of supernatant, 1250 μl of cold methanol were added and was kept overnight at -20 °C. After this procedure, the samples were centrifuged as previously and the pellets were collected and resuspended in 30 μl Laemmli sample buffer, containing 0.1 M Tris-HCl pH: 7.5, 4% SDS, 20% glycerol, 0.004% bromophenol blue. To avoid bisulfate bonding, 0.1 M DTT (DiThioThreitol) was added to each sample. The samples were kept at -20 °C until further use.

SDS-PAGE electrophoresis

Samples were boiled at 55°C - 60°C for 10 minutes and 10 μl of each sample were loaded to the stacking gel containing 4% acrylamide. Electrophoresis was carried out at room temperature at a constant voltage of 85V until the samples reached the separating gel containing 12.5% acrylamide were was carried out at 110V.The device that contained the gel was filled with running buffer.

Immunoblotting

Proteins were separated according to their molecular weight within the separating gel and blotted onto a nitrocellulose membrane (Nitrocellulose membrane Macherey-Nagel, Düren, Germany) by applying a current of 350mA, for 1 hour and 15 minutes, at 4°C. The device that was used for the transfer of proteins was filled with transfer buffer. Blots were incubated in a 3% BSA solution (bovine serum albumin) in order to prevent any non-specific background binding of the primary or the secondary antibody. Then, they were

washed three times with TBST (10 min each wash) and incubated overnight at 4°C, with one of the primary antibodies shown in the table below.

Antibody	Host	Working dilution	Company
ph-Akt	Rabbit	1:1000	Cell Signaling
t-Akt	Rabbit	1:1000	Cell Signaling
CB1R	Rabbit	1:200	Abcam

* Abbreviations: ph = phosphorylated, t = total

To estimate the phosphorylation of Akt, the blots were incubated first with the ph-Akt antibody, then stripped by incubation in mild stripping buffer, blocked again in a 3% BSA solution and finally incubated again with the primary antibody against to total-Akt.
To normalize for protein content the blots were stripped and stained with a rabbit polyclonal antibody raised against GAPDH (1:1000, Cell Signaling), as described previously. Blots were incubated with the peroxidase-conjugated secondary antibody HRP-goat anti-rabbit IgG (1:5000; Invitrogen, Waltham, Massachusetts, USA). Proteins were visualized using the ECL Western blotting kit (LumiSensor Chemiluminescent HRP Substrate Kit, Genscript), and the optical density of the bands in each blot was quantified using the ImageJ Software.
Quantification data for each treatment was expressed as CB1R/GAPDH ratio, in order to show the CB1R protein normalized to the total protein content, or as ph-Akt/total-Akt ratio to express the phosphorylation of Akt.

3.9 Buffers

PBS 50mM (Vf = 100 ml)

0.9gr NaCl
0.2M PB 25ml

PBS 0,1M (Vf = 200 ml)

1,8gr NaCl
0.2M PB 100ml

TBS 0,1M (Vf = 500 ml)

6,055gr Trisma Base
4,5gr NaCl

Gelatin solution (Vf = 1L)

5gr gelatin
0.5gr Chrom Alum (AlCrO3)

30% Sucrose (Vf = 10 ml)

3gr Sucrose
0.1M PB (10ml)

PB 0,2M (Vf = 600 ml)

- *0,2M NaH_2PO_4 (Vf = 100 ml)*

2,4gr NaH_2PO_4 /100ml

- *0,2M K_2HPO_4 (Vf = 500 ml)*

17,42gr K_2HPO_4 /500ml

The two solutions above were mixed to make the final solution PB 0,2M (Vf = 600 ml.

PFA 4% (Vf = 100 ml)

4gr PFA were diluted in 50ml dH_2O in 55◦C and 4 drops of NaOH 4% were added.The final volume was adjusted to 100 ml by adding 50ml PB 0,2M.

Western blot buffers

Separating gel 12.5 %			**Stacking gel 12.5 %**		
	2 Gels	**1 Gel**		**2 Gels**	**1 Gel**
dH_2O	6.25 ml	3.125 ml	**dH_2O**	4.61 ml	2.035 ml
1.5M Tris-HCl pH=8.8	3.75 ml	1.875 ml	**0.5M Tris-HCl pH=6.8**	1.875 ml	0.937 ml
Acrylamide (30%)	7.25 ml	3.625 ml	**Acrylamide (30%)**	1.005 ml	0.502 ml
SDS (20%)	75 µl	37.5 µl	**SDS (20%)**	37.5 µl	18.75 µl
TEMED	7.5 µl	3.75 µl	**TEMED**	7.5 µl	3.75 µl
APS (10%)	75 µl	37.5 µl	**APS (10%)**	37.5 µl	18.75 µl

Running Buffer 10X (Vf = 1L)

25mM Tris-HCl pH:8.3 (30.3gr)
Glycine 144.2gr
SDS 10gr
The running buffer was used at 1X = 100mL of running buffer (10X) + 900mL dH_2O

Transfer Buffer 10X (Vf =1L)

25mM Tris-HCl pH:8.3 (30.3gr)
Glycine 144.2gr
The transfer buffer was used at 1X = 100mL of transfer buffer (10X) + 100mL Methanol + 800mL dH_2O

Laemmli buffer sample buffer 2X (Vf = 50 mL)

5mL 1M Tris-HCl pH:7.4
20 mL SDS 10%
0.1 gr bromophenol blue
10 mL glycerol 100%
15 mL dH_2O

TBS 10X (Vf = 1L)

190 mM Tris-HCl pH:7.4 (23.02 gr)
1.37 mM NaCl (80 gr)
27mM KCl (2.01 gr)

TBST (Vf = 500 mL)

50 mL of TBS 10X
450 mL dH_2O
250 µl Tween-20

1.5M Tris-HCl (pH=8.8): 9.08 gr/50 mL

0.5M Tris-HCl (pH=6.8): 3.02 gr/50mL

Acrylamide 30% (Vf = 100 ml)

29 gr of acrylamide and 1gr of N-N'-methylacrylamide were dissolved in 60 ml of dH_2O. The solution was heated at 37ºC and the volume was adjusted to 100 mL with dH_2O. The pH should be 7 or less.

SDS 20%: 2gr/10mL

APS 10% :1gr/10mL

Mild Stripping buffer (Vf = 100 ml)

1.5 g Glycine
0.1 g SDS
1 ml Tween20
100 ml dH_2O
The pH was adjusted at 2.2.

Harsh Stripping buffer (Vf = 100 ml)

20 ml SDS 10%
12.5 ml TRIS-HCl pH 6.8
67.5 ml dH_2O
0.8 ml β-ME

4. Results

To investigate the effect of subchronic or chronic cannabinoid treatment on the expression of the CB1 receptor in the retina, the synthetic cannabinoid HU-210 was administered in different doses (25, 50 and 100 μg/kg, i.p) and the duration of the treatment lasted 4 or 14 days, respectively. Subsequently, the effect of HU-210 on CB1 receptor downregulation was studied in the animal model of AMPA excitotoxicity, in which HU-210 protected the retina from the toxic insult when it was co-injected with AMPA. Moreover the effect of HU-210 treatment on the PI3K/Akt signaling pathway was also studied.

4.1 Localization of the CB1 receptor in rat retina

To examine the expression of the CB1 receptor in rat retina, a rabbit polyclonal antibody raised against the C-terminal of the receptor was used for immunohistochemical studies. In the control retina, CB1R immunoreactivity was localized robustly in the ganglion cell layer and less in the inner plexiform, inner nuclear and outer plexiform layers. In order to test the specificity of the staining, the primary antibody was omitted, leading to the lack of the immunoreactivity observed in the control tissue (Fig.1A). To confirm the expression of the CB1 receptor in ganglion cells and processes, colocalization studies were performed with beta-tubulin III, a marker for ganglion cells and their processes inner plexiform layer (IPL) CB1R immunoreactivity was colocalized with beta-tubulin immunoreactivity in the ganglion cell layer (GCL), as well as in the IPL (Fig. 1B).

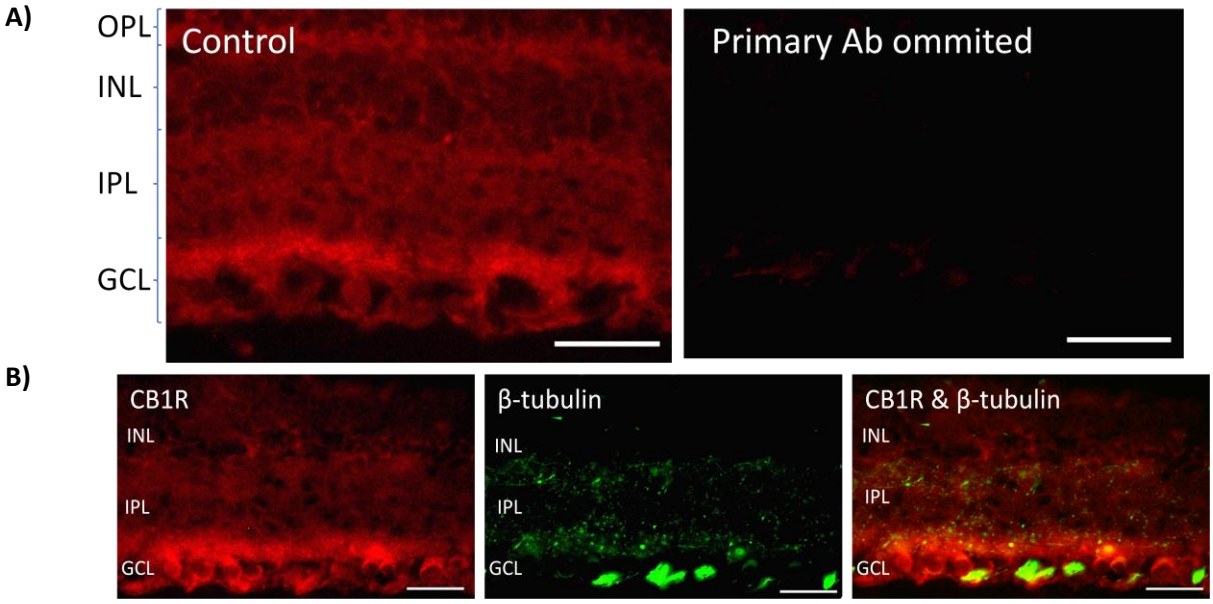

Figure 1: Immunohistochemical studies showing the localization of the CB1R immunoreactivity **A.** CB1R immunoreactivity was found mostly in the GCL and less in the IPL, INL, OPL. Omission of the primary antibody led to the lack of CB1R immunoreactivity. **B.** Colocalization studies of CB1R with β-tubulin III, a marker of ganglion cells. CB1 receptor is colocalized with β-tubulin immunoreactivity in ganglion cells. OPL: outer plexiform layer, INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer. Scale bar: 50μm.

4.2 Acute, subchronic and chronic effects of HU-210 on CB1 receptor expression in rat retina

In order to investigate if the subchronic or chronic cannabinoid treatment leads to downregulation of the CB1 receptor in healthy rat retina, the synthetic cannabinoid HU-210 was administered intraperitoneally (i.p.) in different doses (25, 50 and 100 μg/kg) for 4 (subchronic) and 14 (chronic) days, respectively. To study the acute effects of HU-210, rats were treated with vehicle for 13 days and with one single dose of HU-210 on the 14th day. The lower dose of HU-210, 25μg/kg, did not provide any statistically significant decrease ($p<0.05$ compared to control) in CB1R immunoreactivity, in any of the time points studied (Fig. 2A).

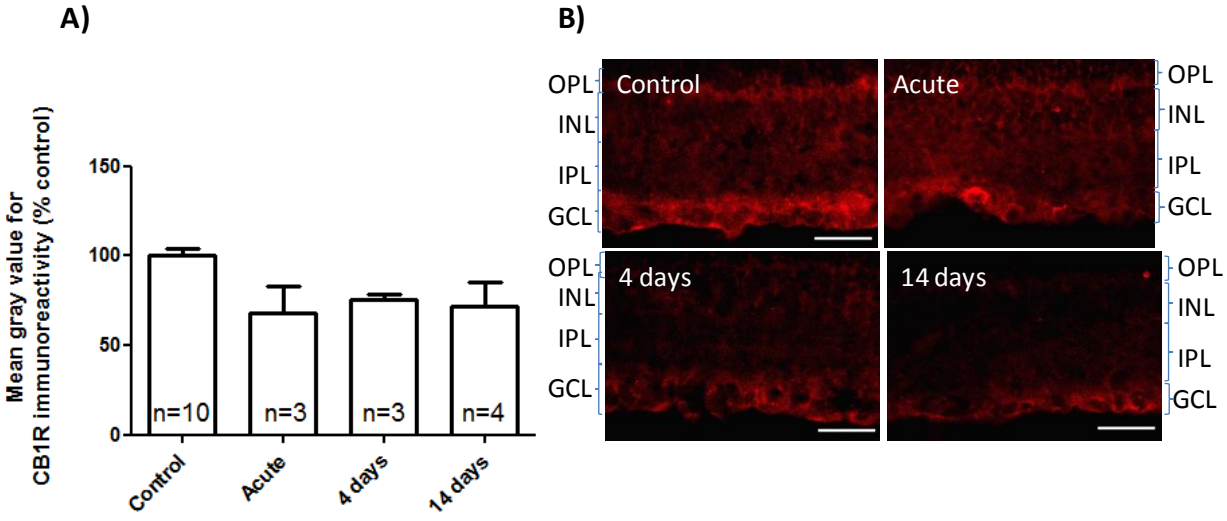

Figure 2: A. Quantification studies for CB1R immunoreactivity in the ganglion cell layer (GCL). HU-210 (25 µg/kg, i.p.) administered intraperitoneally did not affect the immunoreactivity of the CB1R after acute, subchronic (4 days) or chronic (14 days) treatment. **B.** Representative images of CB1R immunoreactivity in the retina of control, acutely, subchronically and chronically treated retinas. Scale bar: 50µm.

\

HU-210 administered at the dose of 50 µg/kg (i.p.) induced a significant reduction of in CB1R immunoreactivity either after 4 or 14 days treatment (*p<0,001 compared to control), while it had no effect when administered acutely (Fig. 3A). In addition, statistically significant differences were found between the acute and 4 or 14 days treated groups (###p<0.001), as well as between 4 and 14 days treatment (##p<0.01).

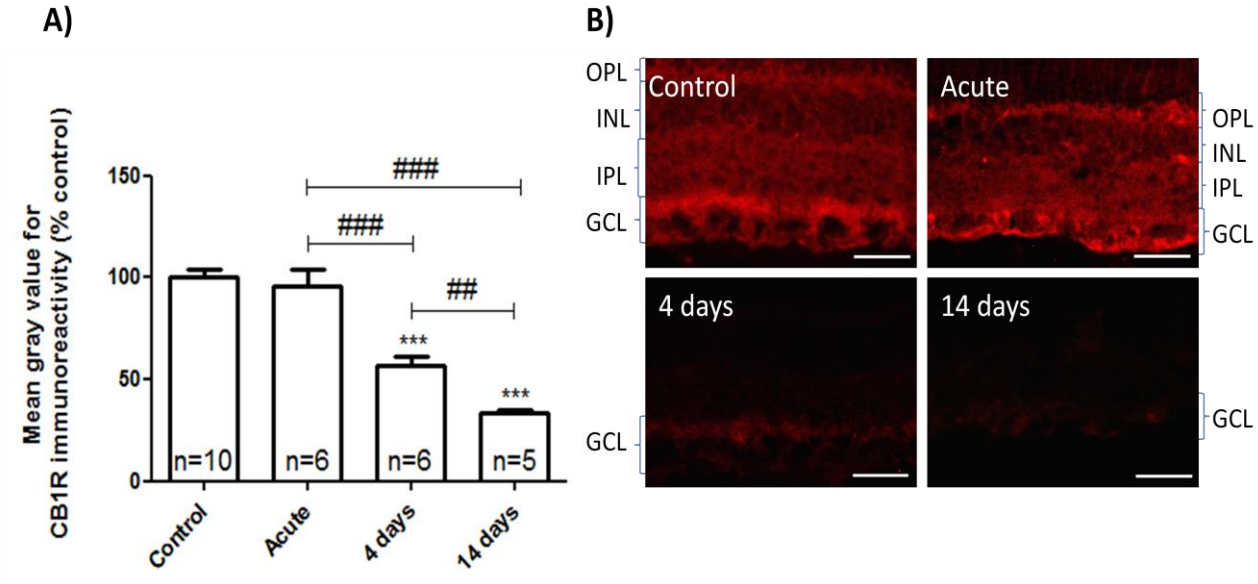

Figure 3: A. Quantification studies for CB1R immunoreactivity in the ganglion cell layer (GCL). HU-210 (50 μg/kg, i.p.) administered intraperitoneally decreased the immunoreactivity of the CB1R after subchronic (4 days) or chronic (14 days) treatment (*p<0.001). Acute administration did not afford any statistically significant decrease in the immunoreactivity. **B.** Images shown represent the reduction in the immunoreactivity observed in retinas of the four groups. Scale bar: 50μm.

The highest dose of HU-210 (100μg/kg) induced a statistically significant reduction in the immunoreactivity of the CB1R after acute (*p<0.05 compared to control), and 4 or 14 days administration (**p<0.01 compared to control) (Fig. 4A).

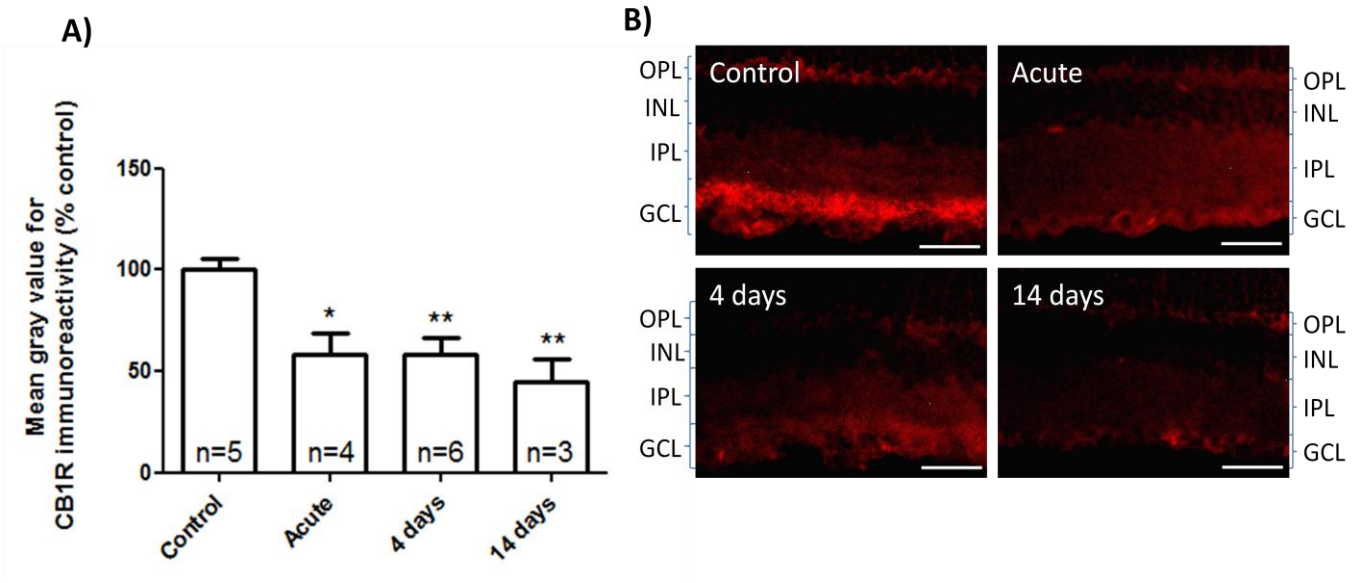

Figure 4: A. Quantification studies for CB1R immunoreactivity in the ganglion cell layer (GCL) after HU-210 (100 μg/kg, i.p.) intraperitoneal administration. HU-210 decreased the immunoreactivity of the CB1R after acute (*p<0.05), subchronic (4 days) or chronic (14 days) treatment (**p<0.01, one-way ANOVA). **B.** Representative images showing the reduction in the immunoreactivity observed in retinas of the four groups. Scale bar: 50μm.

The cumulative data showing the time- and dose-dependet effects of HU210 are presented in Figure 5. The lower dose of 25μg/kg had no statistically significant effect on CB1R immunoreactivity after the acute, 4 or 14 day administration (p>0.05 compared to control). The medium dose of 50 mg/kg led to an attenuation of CB1R immunoreactivity at

subchronic (***p<0.001 compared to control) and chronic (***p<0.001 compared to control) treatments, respectively. No difference in CB1R immunoreactivity was observed between the acute and the control groups (p>0.05). The intraperitoneal administration of HU-210 at the highest dose of 100 µg/kg led to a reduction of CB1 R immunoreactivity in all three groups, namely the acute,subchronic and chronic (*p<0.05, **P<0.01).

So far, these data demonstrate that downregulation of the CB1 receptor occurs in the retina after the acute, subchronic or chronic administration of HU-210 depending on the dose employed. CB1R immunoreactivity was shown to be reduced after subchronic or chronic administration of HU-210 (50 or 100µg/kg), whereas the dose of 100µg/kg also reduced CB1R immunoreactivity in the acute treated animals.

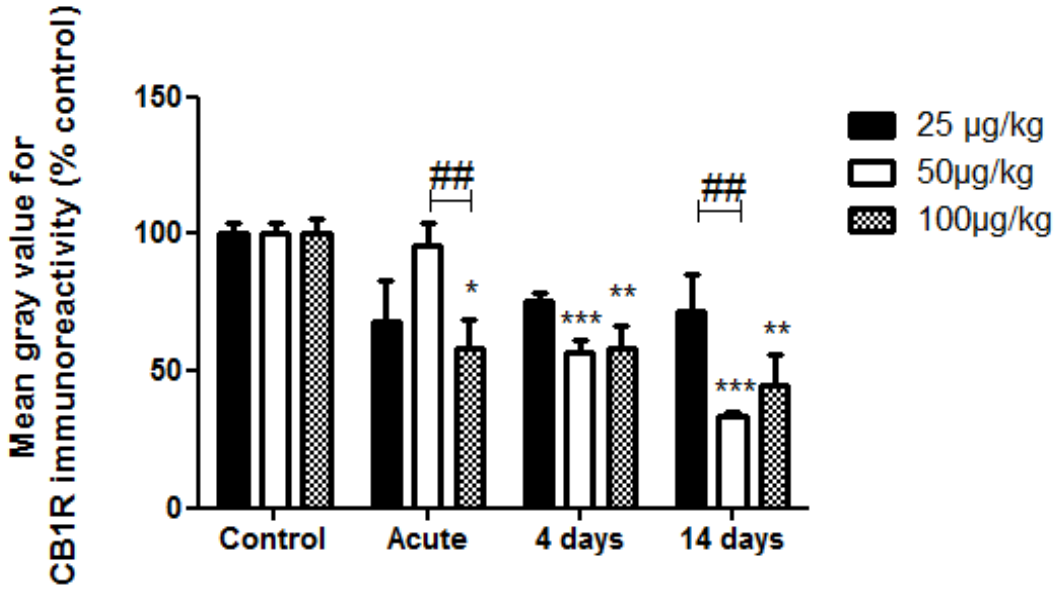

Figure 5: Quantification studies showing the dose-dependent and time-dependent effects of HU-210 administration on CB1R immunoreactivity in the GCL. HU-210 administered either subchronically or chronically induced a dose-dependent reduction in CB1R immunoreactivity. Statistical analysis was performed using two-way ANOVA (*p<0.05, compared to control, #p<0.05, compared to the doses, +p<0.05, compared to subchronic and chronic treatment).

Western blot analysis was also performed and the results confirmed the decrease of CB1R expression in the subchronic and chronic groups. The CB1R was detected as a protein with an approximate 60kD molecular mass. Administration of HU-210 (50µg/kg, i.p.) led to the decrease of the CB1R protein expression after 4 days (*p<0.05 compared to control) or 14

days treatment (**p<0.01 compared to control) (Fig.6). Likewise, the higher dose of HU-210 (100μg/kg) decreased CB1R protein expression either after 4 days (**p<0.01 compared to control) or 14 days treatment (*p<0.05 compared to control) (Fig.7).

The data from western blot analysis are in agreement with the immunohistochemical studies that support the downregulation of the CB1R, as statistically significant difference in the CB1R protein expression was observed in retinal homogenates, after subchronic or chronic administration of HU-210 (50 or 100μg/kg, i.p.).

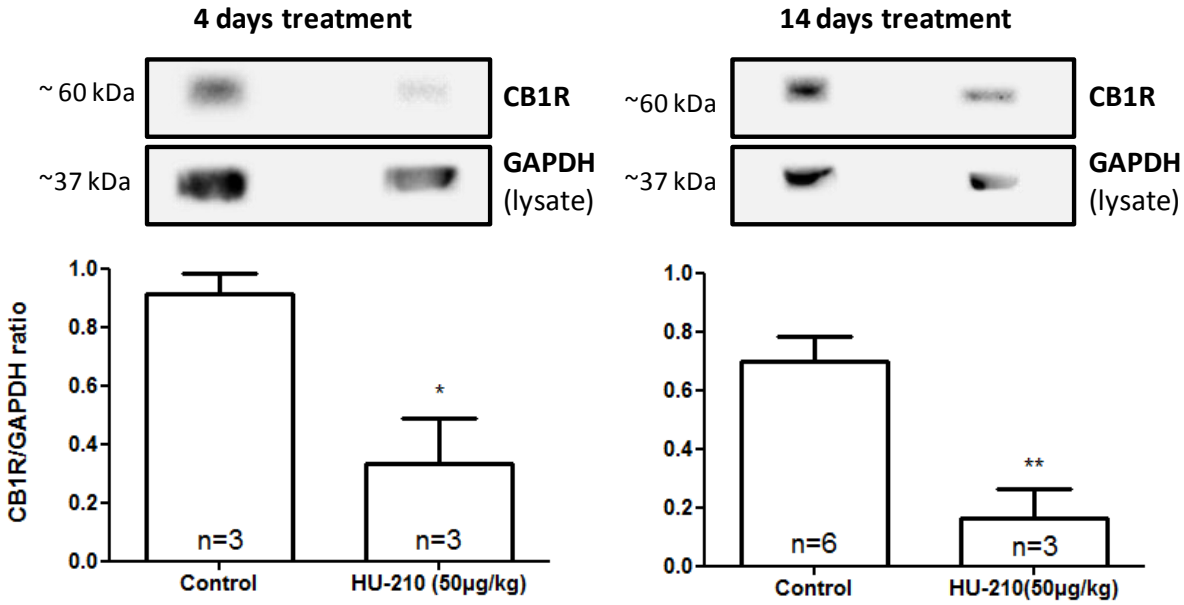

Figure 6: Western blot and quantification analysis for the CB1R protein expression in rat retina. HU-210 (50 μg/kg, i.p) administration decreased the expression of the CB1R protein after 4 days (*p<0.05 compared to control) or 14 days treatment (**p<0.01 compared to control).

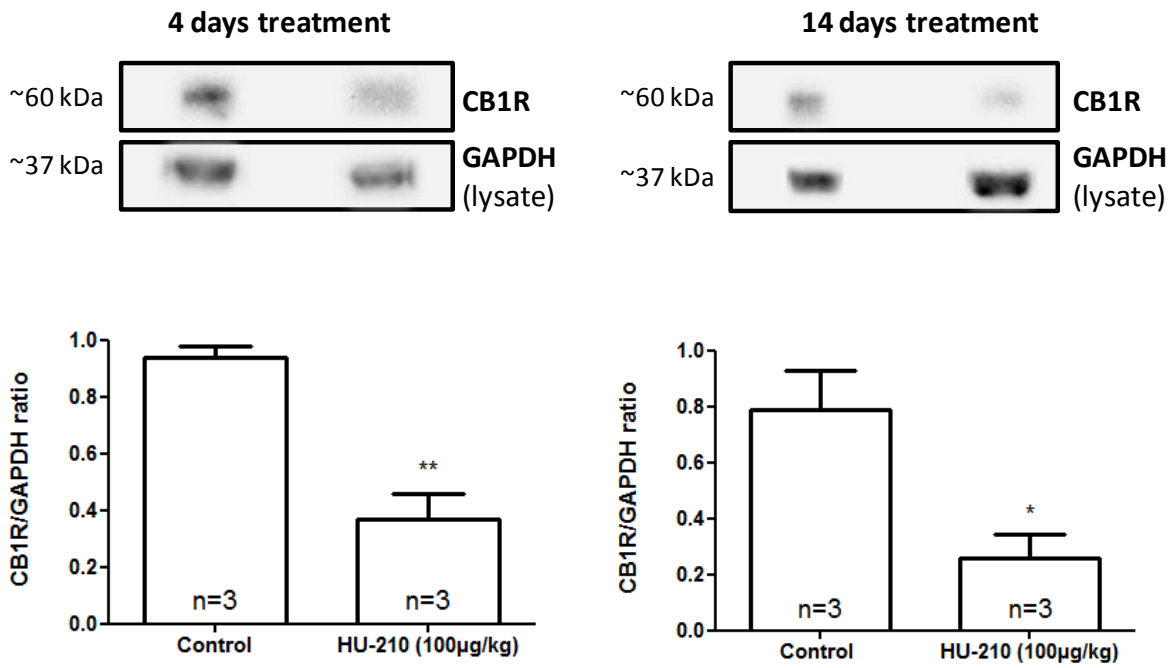

Figure 7: Western blot and quantification analysis for the CB1R protein expression in rat retina. HU-210 (100 µg/kg, i.p) administration decreased significantly the expression of the CB1R protein after 4 days (**p<0.01 compared to control) or 14 days treatment (*p<0.05 compared to control).

Effect of HU-210 on PI3K/Akt signaling pathway

Endogenous and synthetic cannabinoids, as HU-210, have been shown to exert their effects through the CB1 receptor. Moreover they provided neuroprotection to the retina via the activation of PI3K/Akt prosurvival pathway (Kokona & Thermos, 2015), thus the effect of the chronic cannabinoid administration on PI3K/Akt activation was studied.

To examine the effect of the HU-210 administration on the PI3K/Akt signaling pathway, the ratio of the phosphorylated Akt to the total Akt protein was quantified by western blot analysis (Fig. 8). HU-210 (100 µg/kg, i.p.) administration decreased the phospho-Akt/total-Akt ratio after 14 days treatment (*p<0.05).

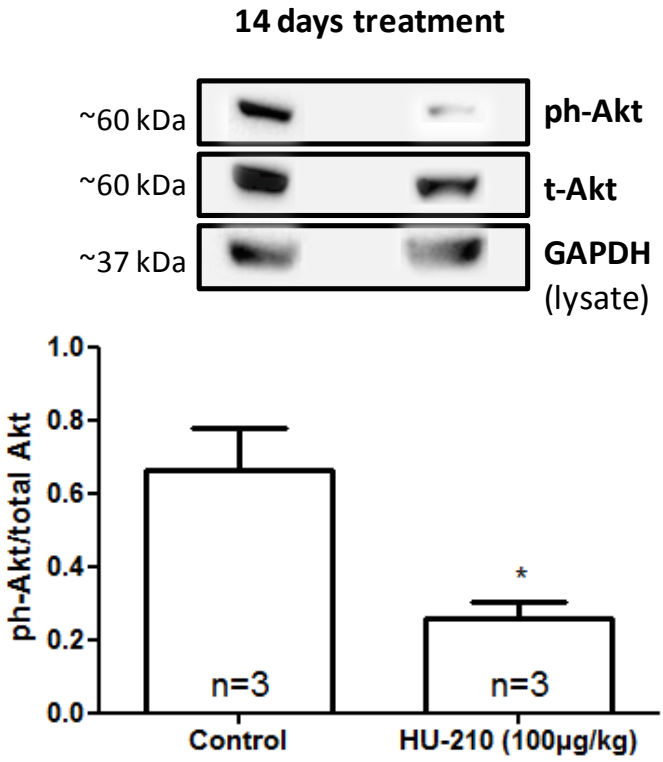

Figure 8: Western blot and quantification analysis for Akt protein phosphorylation in rat retina. HU-210 (100µg/kg, i.p.) administration decreased significantly the phosphorylation of the Akt protein after 14 days treatment (*p<0.05 compared to control).

In summary, the synthetic cannabinoid HU-210 was found to induce a reduction in the expression of the CB1 receptor in rat retina, as it decreased CB1R immunoreactivity and CB1R protein expression, after subchronic or chronic administration. Also, when injected acutely in the high dose of 100µg/kg, it decreased the immunoreactivity of the CB1 receptor. Additionally, HU-210 (100µg/kg) chronic administration decreased the phosphorylation of Akt protein and therefore the activation of PI3K/Akt pathway.

4.3 Neuroprotective effects of HU-210 against AMPA excitotoxicity in rat retina

HU-210 provides neuroprotection in the retina against AMPA excitotoxicity *in vivo* (Kokona & Thermos, 2015). In order to examine the effect of the subchronic orchronic administration of HU-210 in this model, we initially examined the neuroprotective actions of HU-210 when administered intravitreally. Intravitreal administration of AMPA (8.4mM) led to the loss of bNOS expressing amacrine cells in the INL and displaced

amacrine cells in the GCL (***p<0.001 compared to control) (Fig. 9A). Co-administration of AMPA and HU-210 (10^{-6} M) reduced the loss of bNOS immunoreactive cells (#p<0.05 compared to AMPA treated retinas), in agreement to Kokona & Thermos (2015). CB1R immunoreactivity in the GCL, IPL and INL, was not affected by the administration of AMPA or by the co-administration of AMPA with HU-210 (10^{-6} M) (Fig. 9B).

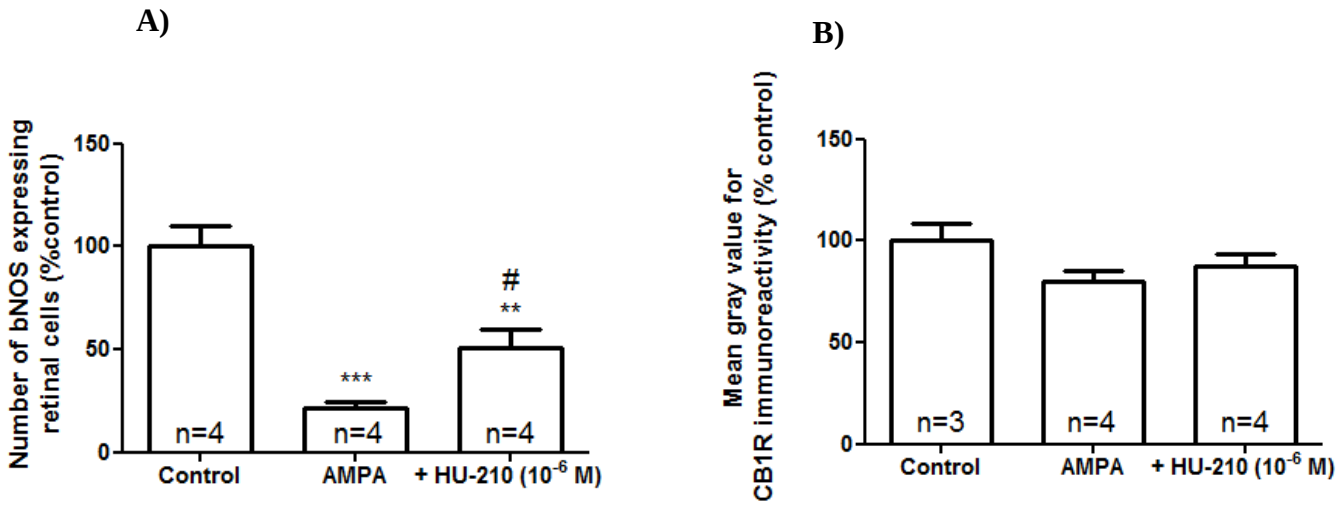

Figure 9: A. Quantification studies of bNOS expressing amacrine cells. Administration of AMPA (8.4 mM) decreased the number of bNOS immunoreactive cells (***p<0.001 compared to control) and the co-injection of HU-210 (10^{-6} M) increased the number bNOS expressing amacrine cells comparing to AMPA (#p<0.05), but did not manage to reach the control levels (**p<0.01 compared to control). **B.** Quantification studies for CB1R immunoreactivity. Neither AMPA nor its co-administration with HU-210 have any statistically significant effect on CB1R immunoreactivity. Statistical analysis was performed using one-way ANOVA (p>0.05).

4.4 Effect of HU-210 subchronic treatment on the expression of the CB1 receptor in the model of AMPA excitotoxicity

In order to study if the subchronic or chronic administration of HU-210 affords neuroprotection in the model of AMPA excitotoxicity, HU-210 (50μg/kg) was administered for 4 days. The dose of 50mg/kg and the subchronic treatment of HU-210 were chosen for this set of experiments because our previous data in control animals showed no difference in CB1R immunoreactivity between the 50μg/kg and 100μg/kg dose in the subchronic and chronic groups. Similar results were obtained using western blot analysis (shown above).

Intravitreal administration of AMPA (8.4 mM) led to the decrease of bNOS expressing amacrine cells (***$p<0.001$ compared to control) in INL and GCL (Fig. 10A), while it did not affect the CB1R immunoreactivity in the INL, IPL and GCL (Fig. 10B). HU-210 (50μg/kg, i.p.) subchronic (4 days) administration did not protect the bNOS expressing amacrine cells (***$p<0.001$, compared to control) (Fig. 10A). CB1R immunoreactivity was decreased in a statistically significant manner (**$p<0.01$, compared to control, ##$p<0.01$, comparing to AMPA) (Fig. 10B).

These results support that downregulation of the CB1 receptor is influencing the neuroprotective actions of HU-210 against AMPA excitotoxicity.

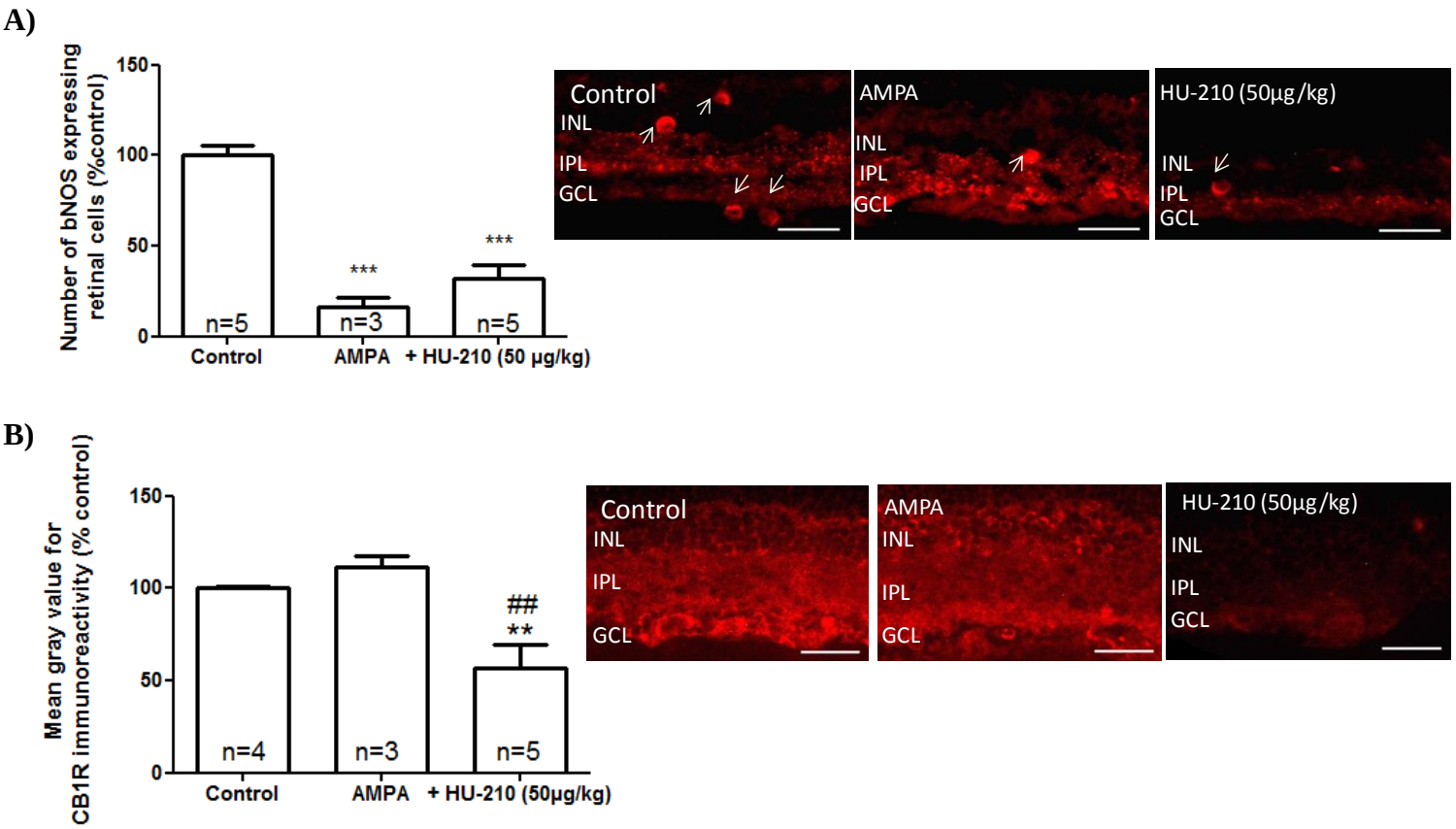

Figure 10: A. Quantification studies and representative images for bNOS expressing amacrine cells. Administration of AMPA decreased the number of bNOS immunoreactive cells (***p<0.001 compared to control) and HU-210 (50 µg/kg, i.p.) subchronic (4 days) treatment, did not reverse the cell loss (***p<0.001 compared to control, p>0.05 compared to AMPA). **B.** CB1R immuneactivity was reduced significantly after HU-210 subchronic administration (**p<0.01, compared to control, ##p<0.01compared to AMPA). Scale bar: 50µm.

5. Discussion

The results of the present study provide information about the function of CB1 receptors in adult retina and provide answers to the question "Is the CB1 receptor downregulated after chronic exposure of the agonist HU-210 in the retina". CB1 receptor downregulation was observed in healthy retina, as well as in ischemic retina using the model of AMPA excitotoxicity.

The CB1 receptor is expressed in the ganglion cell, inner plexiform, inner nuclear and outer plexiform layers (GCL, IPL, INL, OPL, respectively) in rat retina. These data are in agreement with Zabouri et al. (2011) who reported that the CB1 receptor is expressed in all layers in the adult rat retina and moreover it is largely expressed in cone photoreceptors, horizontal, in most amacrine and in ganglion cells. The expression of the receptor in the two plexiform layers and in amacrine and ganglion cells and axons in rat retina is further supported by Straiker et al. (1999). In the present study the expression of CB1 receptor in ganglion cells was confirmed by co-localization of CB1R immunoreactivity with β-tubulin III immunoreactivity, a marker for ganglion cells. Zabouri et al. (2011) also showed co-localization of CB1R with Brn3 and syntaxin immunoreactivities, which are cell-type-specific markers for ganglion cell and retinal interneurons (horizontal and amacrine cells), respectively. However, in the literature, apart from this study, there is little information for the identity of CB1R expressing retinal neurons in the INL. Therefore further investigation for the expression pattern of the CB1 receptor in the INL is necessary, in order to elucidate the role of the endocannabinoid system in retinal function and in the putative neuroprotective effects in the retina.

In this study, CB1 immunoreactivity was examined in retinas of rats treated with the synthetic cannabinoid HU-210 (CB1/CB2 agonist) under acute, subchronic and chronic conditions in order to investigate whether this treatment will lead to the downregulation of the receptor. To date, there is no information on the agonist induced downregulation of the CB1 receptor in the retina.

The subchronic or chronic administration of HU-210 was shown to induce downregulation of the CB1 receptor in a dose-dependent manner. The lower dose of HU-210 (25μg/kg)

employed did not affect CB1 receptor expression when administered in any of the time points studied, nor did the intermediate dose of 50μg/kg when administered acutely. Only the highest dose (100μg/kg) induced a statistically significant decrease in CB1 receptor expression in all conditions studied (Fig.5). In a relatively recent study by Dalton et al. (2009, 2010) the effect of CB1 receptor density in several regions of rat brain was examined by autoradiographic binding studies, after subchronic and chronic treatment with HU-210, administered in the same doses as the present study. A dose-dependent decrease in binding was observed in all regions examined after 4 or 14 days treatment. Moreover a single injection of HU-210 in the highest dose of 100μg/kg induced a decrease in binding to CB1 receptor in caudate putamen and hippocampus in adult and adolescent rat brain. These data are in agreement with the findings of the present study that was performed in rat retina.

Western blot studies performed in retinas of rats treated with HU-210 (50, 100μg/mg, i.p.) in subchronic and chronic conditions provided data that agree fully with the results obtained from the immunohistochemical studies.

It is known that cannabinoids exert their actions via the activation of their receptors and the subsequent activation of downstream signaling pathways. Activation of the CB1 receptor by HU-210 led to the phosphorylation of the Akt protein but not ERK1/2 kinases in oligodendrocyte progenitors (Molina-Holgado et al., 2002). The results from the present study showed that the subchronic and chronic intraperitoneal administration of HU-210 reduced the phosphorylation of Akt protein in control animals. This effect was expected, as CB1 receptors are downregulated by this treatment, as mentioned above, and therefore the activation of PI3K/Akt signaling pathway is reduced.

The results of the present study examining the effect of subchronic or chronic administration of the cannabinoid agonist HU-210 on CB1 receptor levels in the retina strongly suggest that the receptor is downregulated in both conditions.

Previous studies have reported the neuroprotective actions of endocannabinoids and synthetic cannabinoids (HU-210) in animal model of retinopathies and suggested that these agents are putative therapeutics in retinal disease (Nucci et al., 2007, Lax et al., 2014, Kokona and Thermos, 2015). According to Kokona & Thermos (2015), the endogenous cannabinoid anandamide was found to afford neuroprotection to the retina against AMPA excitotoxicity. Neuroprotective effects were also observed in the same model by 2-AG and

inhibitors of the metabolic enzymes for AEA and 2-AG, FAAH and MGL, respectively (Kokona, unpublished data). Inhibition of these enzymes leads to an increase of the AEA and 2-AG levels. Naidoo et al. (2012) also reported that inhibition of these enzymes provides protection against excitotoxic brain injury.

Taken into account that retinal diseases such as glaucoma and diabetic retinopathy are chronic diseases it is very important to assess the neuroprotective properties of these agents when administered chronically in models of retinopathy. In the present study, we examined the effect of the subchronic and chronic administration of HU210 in its neuroprotective properties in the AMPA excitotoxicity model.

HU-210, when administered intravitreally and in conjunction with AMPA, was found to afford neuroprotection in rat retina against AMPA excitotoxicity and this neuroprotection was shown to be mediated by the CB1 receptor (Kokona & Thermos, 2015). Our present data showed that the co-injection of HU-210 (10^{-6} M) with AMPA prevented the loss of bNOS immunoreactive amacrine cells, in agreement with the study cited previously. The CB1 receptor immunoreactivity was not affected by the AMPA excitotocity insult.

The mechanism involved in the HU-210 neuroprotection of the retina against AMPA excitotoxicity was shown to be due to the activation of the CB1 receptor and subsequent activation of PI3K/Akt signaling pathway (Kokona & Thermos, 2015). These findings were further supported by Molina-Holgado et al. (2005) who showed that HU-210 exerted neuroprotective effects against S-AMPA-induced excitotoxicity in primary cortical neurons by activating the PI3K/Akt signaling pathway.

The subchronic administration of HU-210 led to an attenuation of its neuroprotective actions against AMPA excitoxicity in the retina due to the downregulation of the CB1 receptor, in agreement with the data observed in control animals.

A previous report alluded to the possible down regulation of the CB1 receptor after the administration of inhibitors of FAAH, the metabolic enzyme of AEA (Nucci et al., 2007). FAAH inhibitors increase retinal levels of AEA and as such could be possible neuroprotectants in the retinal model of high intraocular pressure (IOP) ischemia-reperfusion. Their data showed that the inhibition of the metabolic enzymes did not afford neuroprotection, and the authors suggested that the CB1 receptor was downregulated as was shown by the reduction of its expression and binding capacity.

The mechanism via which the CB1 receptor is downregulated has not been elucidated. It has been reported that HU-210 and other synthetic cannabinoids can cause rapid internalization of the CB1 receptor and this internalization occurs via clathrin-coated pits, but it does not require active G protein α_i, α_o, or α_s subunits (Hsieh et al., 1999). In addition, another study suggested that CB1 receptor internalization can be mediated by clathrin-coated pits but also via caveolae (Keren & Sarne, 2003).

There are several studies in the literature that have examined the chronic effects of Δ^9-THC and have shown that its repeated administration induces profound tolerance that correlates with desensitization and downregulation of CB1 cannabinoid receptors in brain (Sim-Selley et al., 2002, 2006). Willoughby et al. (1997) showed that repeated administration of anandamide produces less adaptive changes to the CB1 receptor compared to THC and this phenomenon could be correlated with the fact that anandamide is rapidly metabolized by its metabolic enzymes. $FAAH^{-/-}$ mice were shown to display differential tolerance, dependence, and cannabinoid receptor adaptation after THC and anandamide administration. In contrast to the report by Nucci et al. (2007) mentioned above, this study showed that AEA produced less cellular adaptation and associated dependence and less tolerance to its own effects than administration of THC (Falenski et al., 2010). In addition, in a study that examined the analgesic effects of the chronic use of 2-AG a significant decrease in CB1 receptor density and function was reported in $MAGL^{-/-}$ mice or mice chronically treated with the MAGL inhibitor JZL184, (Schlosburg et al., 2010). These findings suggested that sustained elevation of 2-AG in brain produced by either genetic deletion or chronic pharmacological blockade of MAGL caused impaired endocannabinoid-dependent synaptic plasticity, and desensitization of brain CB1 receptors, in contrast to the results from Falenski et al. (2010). The results of these studies suggest that brain CB1 receptors undergo different adaptations in response to the elevated levels of the two principal endocannabinoids, 2-AG and anandamide. It appears that FAAH inhibitors may be more efficacious pharmacological targets, than MAGL inhibitors, and will afford a greater therapeutic potential. Therefore, pharmacotherapies targeting endocannabinoid metabolic enzymes are less likely to promote tolerance and dependence than targeting CB1 receptor agonists.

Cutando et al. (2009) correlated CB1 receptor downregulation with inflammatory responses and CB2 upregulation. The subchronic administration of Δ^9-THC increased neuroinflammatory markers in cerebellum. More specifically the recruitment of microglial

cells was observed, and the mRNA levels of the cytokine IL-1β and CB2 receptor were increased. There is evidence that in inflammatory conditions in rat retina, microglial activation is observed, and found to be mediated by CB2 receptors (Slussar et al., 2013)

Conclusions

The findings of the present study strongly suggest that the synthetic cannabinoid HU-210 when administered chronically leads to downregulation of the CB1 receptor. Therefore, caution should be used when using it as a neuroprotectant and putative therapeutic in chronic retinal diseases. Further studies are essential to investigate the chronic effects of anandamide and FAAH inhibitors on the downregulation of the CB1 receptor in control retina and in models of retinopathy since these agents may be more efficacious as therapeutics as suggested from the studies mentioned above. In addition, the correlation of the CB1 receptor downregulation with neuroinflammatory responses and the possible upregulation of CB2 receptor should be investigated in retina. Further studies are essential in order to recommend the cannabinoids as potential therapeutics for retinal diseases.

6. References

Andrés, N., Malpesa, Y., Rodríguez, M.J., Mahy, N., 2003. Low Sensitivity of Retina to AMPA-Induced Calcification. *J. Neurosci. Res.* 72: 543-548.

Antonetti, D.A., Klein, R., Gardner, T., 2012. Mechanisms of disease in Diabetic Retinopathy. *N. Engl. J. Med.* 366: 1227-1239.

Bakshi, K., Mercier, R.W., Pavlopoulos, S., 2007. Interaction of a fragment of the cannabinoid CB1 receptor C-terminus with arrestin-2. *FEBS Lett.* 581: 5009–5016.

Beilin, M., Neumann, R., Belkin, M., Green, K., Bar-Ilan, A., 2000. Pharmacology of the intraocular pressure lowering effect of systemic dexanabinol (HU- 211), a non-psychotropic cannabinoid. *J. Ocul. Pharmacol. Ther.* 16: 217–229.

Berdyshev, E.V., 2000. Cannabinoid receptors and the regulation of immune response, *Chem. Phys. Lipids.* 108: 169–190.

Beltramo, M., Stella, N., Calignano, A., Lin, S.Y., Makriyannis, A., Piomelli, D., 1997. Functional Role of High-Affinity Anandamide Transport, as Revealed by Selective Inhibition. *Science* 277: 1094-1097.

Bisogno, T., Maurelli, S., Melck, D., Petrocellis, L. De, Di Marzo, V., 1997. Biosynthesis , Uptake , and Degradation of Anandamide and Palmitoylethanolamide in Leukocytes. *J. Biol. Chem.*, 272: 3315–3323.

Bisogno, T., Delton-Vandenbroucke, I., Milone, A., Lagarde, M., Di Marzo, V., 1999. Biosynthesis and inactivation of N-arachidonoylethanolamine (anandamide) and N-docosahexaenoylethanolamine in bovine retina. *Arch Biochem Biophys*. 3702: 300-307.

Bisogno, T., Howell, F., Williams, G., Minassi, A., Cascio, M.G., Ligresti, A., Matias, I., Schiano-Moriello, A., Paul, P., Williams, E.J., Gangadharan, U., Hobbs, C., Di Marzo, V., Doherty, P., 2003. Cloning of the first sn1-DAG lipases points to the spatial and temporal regulation of endocannabinoid signaling in the brain. *The Journal of Cell Biology* 163: 463-468.

Bisogno, T., Oddi, S., Piccoli, A., Fazio, D., Maccarrone, M., 2016. Type-2 cannabinoid receptors in neurodegeneration. *Pharmacol. Res.* 111: 721-730.

Bonhaus, D.W., Chang, L.K., Kwan, J., Martin, G.R., 1998. Dual Activation and Inhibition of Adenylyl Cyclase by Cannabinoid Receptor Agonists: Evidence for Agonist-Specific Trafficking of Intracellular Responses. *J.Pharmacol. Exp. Therap.* 287: 884–888.

Bouaboula, M., Poinot-Chazel, C., Bourriet, B., Canat, X., Calandra, B., Rinaldi-Carmona, M., Le Fur, G., Casellas, P., 1995. Activation of mitogen-activated protein kinases by stimulation of the central cannabinoid receptor CB1. *Biochem. Journal.* 312: 637-641.

Bouaboula, M., Poinot-Chazel, C., Marchand, J., Canat, X., Bourrié, B., Rinaldi-Carmona, M., Calandra, B., Le Fur, G., Casellas, P., 1996. Signaling pathway associated with stimulation of CB2 peripheral cannabinoid receptor. Involvement of both mitogen-activated protein kinase and induction of Krox-24 expression. *Eur J Biochem.* 237(3):704-11.

Bouskila, J., Burke, M. W., & Zabouri, N., 2012. Expression and localization of the cannabinoid receptor type 1 and the enzyme fatty acid amide hydrolase in the retina of vervet monkeys. *Neurosci*. 202: 117–130.

Bouskila, J., Javadi, P., Casanova, C., Ptito, M., Bouchard, J.F., 2013. Muller Cells Express the Cannabinoid CB2 Receptor in the Vervet Monkey Retina. *J. Comp. Neurol.* 521: 2399-2415.

Boycott, B.B, Hopkins, J.M. 1981. Microglia in the retina of monkey and other mammals: its distinction from other types of glia and horizontal cells. *Neurosci.* 6: 679-688.

Brusco, A., Tagliaferro, P., Saez, T., Onaivi, E.S., 2008. Postsynaptic Localization of CB2 Cannabinoid Receptors in the Rat Hippocampus. *Synapse* 62: 944-949.

Buckley, N.E., Hansson, S., Harta,G., Mezey, É., 1998. Expression of the CB1 and CB2 receptor messenger RNAs during embryonic development in the rat. *Neurosci.* 82: 1131-1149.

Cécyre, B., Zabouri, N., Huppé-Gourgues, F., Bouchard, J.F., Casanova, C., 2013. Roles of Cannabinoid Receptors Type 1 and 2 on the Retinal Function of Adult Mice. *Invest. Ophthalmol. Vis. Sci.* 54: 8079-8090.

Cécyre, B., Thomas, S., Ptito, M., Casanova, C., Bouchard, J.F., 2014. Evaluation of the specificity of antibodies raised against cannabinoid receptor type 2 in the mouse retina. *Schmiedeb. Arch. Pharmacol.* 387: 175-184.

Chakrabarti, A., Onaivi, E.S., Chaudhuri, G., 1995. Cloning and sequencing of a cDNA encoding the mouse brain type cannabinoid receptor protein. *DNA Sequence* 5: 385–388.

Chen, Q., Olney, J.W., Lukasiewicz, P.D., Almli, T., Romano, C., 1998. Ca^{2+}-Independent Excitotoxic Neurodegeneration in Isolated Retina, an Intact Neural Net: A Role for Cl^- and Inhibitory Transmitters. *Mol. Pharmacol.* 53: 564-572.

Chen, J., Matias, I., Dinh, T., Lu, T., Venezia, S., Nieves, A., Woodward, D., Di Marzo, V., 2005. Finding of endocannabinoids in human eye tissues: Implications for glaucoma. *Biochem. Biophys. Res. Com.* 330: 1062-1067.

Cutando, L., Busquets-Garcia, A., Puighermanal, E., Gomis-González, M., Delgado-García, J. M., Gruart, A., Ozaita, A. 2013. Microglial activation underlies cerebellar deficits produced by repeated cannabis exposure, *J. Clin. Invest.* 123: 2816-2831.

Dalton, V. S., Wang, H., Zavitsanou, K., 2009. HU210-Induced Downregulation in Cannabinoid CB1 Receptor Binding Strongly Correlates with Body Weight Loss in the Adult Rat. *Neurochem. Res.* 34:1343–1353.

Dalton, V. S., Zavitsanou, K., 2010. Cannabinoid Effects on CB1 Receptor Density in the Adolescent Brain : An Autoradiographic Study Using the Synthetic Cannabinoid HU210. *Synapse* 64: 845–854.

Demuth, D.G., Molleman, A., 2006. Cannabinoid signaling. *Life Sci.*78: 549-563.

De Petrocellis L, Cascio MG, Di Marzo V., 2004. The endocannabinoid system: a general view and latest additions. *Br. J. Pharmacol.* 141: 765–774.

De Petrocellis, L., Di Marzo, V., 2010. Non-CB1, non-CB2 receptors for endocannabinoids, plant cannabinoids, and synthetic cannabimimetics: focus on G-protein-coupled receptors and transient receptor potential channels. *J. Neuroimmun. Pharmacol.* 5: 103–121.

Devane, W., Dysarz, F., Johnson, M., Melvin, L., Howlett, A., 1988. Determination and characterization of a cannabinoid receptor in rat brain. *Mol. Pharmacol.* 34: 605-613.

Devane, W., Hanus, L., Breuer, A., Pertwee, R., Stevenson, L., Griffin, G., Gibson, D., Etinger, A., Mechoulam, R., 1992. Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science* 258: 1946-1949.

De Vry, J., Jentzsch, K., Kuhl, E., Eckel, G., 2004. Behavioral effects of cannabinoids show differential sensitivity to cannabinoid receptor blockade and tolerance development. *Behav. Pharmacol.* 15:1–12.

Di Marzo, V., Fontana, A., Cadas, H., Schinelli, S., Cimino, G., Schwartz, J.C., Piomelli, D., 1994. Formation and inactivation of endogenous cannabinoid anandamide in central neurons. *Nature*, 372: 686–691.

Di Marzo V., Melck D., Bisogno T., De Petrocellis L. 1998. Endocannabinoids: endogenous cannabinoid receptor ligands with neuromodulatory action. *Trends Neurosci* 21: 521–528.

Di Marzo V. 1999. Biosynthesis and inactivation of endocannabinoids: relevance to their proposed role as neuromodulators. *Life. Sci.* 65: 645–655.

Di, Marzo, V., De Petrocellis, L., 2012. Why do cannabinoid receptors have more than one endogenous ligand ? *Phil. Trans. R. Soc. B* 367: 3216-3228.

Dinh, T.P., Freund, T.F., Piomelli, D., 2002. A role for monoacylglyceride lipase in 2-arachidonoylglycerol inactivation. *Chemistry and Physics of Lipids* 121: 149-158.

El-Remessy, A., Behzadian, M., Abou-Mohamed, G., Franklin, T., Caldwell, R., Caldwell, R., 2003. Experimental diabetes causes breakdown of the blood-retina barrier by a mechanism involving tyrosine nitration and increases in expression of vascular endothelial growth factor and urokinase plasminogen activator receptor. *Am. J. Pathol.* 162: 1995–2004

Falenski, K.W., Thorpe, A.J., Schlosburg, J.E., Cravatt, B.F., Abdullah, R.A., Smith, T.H., Sim-selley, L.J., 2010. $FAAH^{-/-}$ Mice Display Differential Tolerance, Dependence, and Cannabinoid Receptor Adaptation after Δ^9-Tetrahydrocannabinol and Anandamide Administration. *Neuropsychopharmacol.* 35: 1775–1787.

Feledziak, M., Lambert, D.M., Marchand-Brynaert, J., Muccioli, G.G., 2012. Inhibitors of the endocannabinoiddegrading enzymes, or how to increase endocannabinoid's activity by preventing their hydrolysis. *Recent Pat. CNS Drug Discov.* 7: 49–70.

Galiegue, S., Mary, S., Marchand, J., Dussossoy D., Carrière, D, Carayon, P, Bouaboula M., Shire, D., Le Fur, G., Casellas, P. 1995. Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. *Eur. J. Biochem*. 232: 54-61.

Gaoni, Y., Mechoulam, R., 1964. Isolation, Structure, and Partial Synthesis of an Active Constituent of Hashish. *J. Am. Chem. Soc.* 86: 1646–1647.

Gomez del Pulgar, T., Velasco, G., Guzman, M., 2000. The CB1 cannabinoid receptor is coupled to the activation of protein kinase B/Akt. *Biochemical Journal* 347: 369-373.

Golech, S.A., McCarron, R.M., Chen, Y., Bembry, J., Lenz, F., Mechoulam, R., Shohami, E., Spatz, M., 2004. Human brain endothelium: coexpression and function of vanilloid and endocannabinoid receptors. *Mol. Brain Res.* 132*:* 87-92.

Gong, J.P., Onaivi, E.S., Ishiguro, H., 2006. Cannabinoid CB2 receptors: Immunohistochemical localization in rat brain. *Brain Res*. 107: 10-23.

Gowran, A., Noonan, J., Campbell, V.A., 2011. The Multiplicity of Action of Cannabinoids: Implications for Treating Neurodegeneration. *CNS Neurosci. Ther.* 17: 637-644.

Griffin, G., Wray, E.J, Tao, Q., McAllister, S.D., Rorrer, W.K., Aung, M.M., Martin, B.R., Abood, M.E., 1999. Evaluation of the cannabinoid CB2 receptor-selective antagonist, SR144528: further evidence for CB2 receptor absence in the rat central nervous system. *Eur. J. Pharmacol*. 377: 117-125.

Grueter, B.A., Brasnjo, G., Malenka, R.C., 2010. Postsynaptic TRPV1 triggers cell type-specific long-term depression in the nucleus accumbens. *Nat. Neurosci.* 13: 1519–1525.

Grunder, T., Kohler, K., Kaletta, A., Guenther, E., 2000. The Distribution and Developmental Regulation of NMDA Receptor Subunit Proteins in the Outer and Inner Retina of the Rat. *J. Neurobiol* 44: 333-342.

Han, J., Kesner, P., Metna-Laurent, M., Duan, T., Xu L., Georges, F., Koehl, M., Abrous, D., Mendizabal-Zubiaga, J., Grandes, P., Liu, Q., Bai, G., Wang, W., Xiong, L., Ren, W., Marsicano, G., Zhang, X., 2012. Acute cannabinoids impair working memory through astroglial CB1 receptor modulation of hippocampal LTD. *Cell* 148: 1039–1050.

Hare, W.A., Wheeler, L., 2009. Experimental Glutamatergic Excitotoxicity in Rabbit Retinal Ganglion Cells: Block by Memantine. *Invest. Ophthalmol. Vis. Sci.* 50: 2940-2948.

Hare, W.A., Dong, C.J., Wheeler, L., 2011. Excitotoxic Injury to Retinal Ganglion Cells. *Glaucoma – Basic and Clinical Concepts*, Rijeka, Croatia, InTech.

Harwerth, R.S., Quigley, H.A., 2006. Visual field defects and retinal ganglion cell losses in patients with glaucoma. *Arch. Ophthalmol.*124: 853-859.

Hepler, R.S., Frank, I.R., 1971. Marihuana smoking and intraocular Pressure. *J. Am. Med. Assoc.* 217: 1392.

Hilfer, R.S., Yang, J.B., 1980. Ocular size and Shape Regulation during Development. *Springer – Verlag*, New York.

Hollister, L.E, 1971. Hunger and appetite after single doses of marihuana, alcohol, and dextroamphetamine. *Clin. Pharmacol. Ther.*12: 44-49.

Howlett A.C., Flemming, R., 1984. Cannabinoid inhibition of adenylate cyclase. Pharmacology of the response in neuroblastoma cell membranes. *Mol. Pharmacol.* 26:532-538.

Howlett, A.C., Champion, T.M., Wilken, G.H., Mechoulam, R., 1990. Stereochemical effects of 11-OH-delta 8-tetrahydrocannabinoldimethylheptyl to inhibit adenylate cyclase and bind to the cannabinoid receptor. *Neuropharmacol.* 29:161–165.

Howlett, H.C., 2002. The cannabinoid receptors. *Prostaglandins & other Lipid Mediators*, 68-69, 619-631.

Howlett, A.C., Breivogel, C.S., Childers, S.R., Deadwyler, S.A., Hampson, R.E., Porrino, L.J., 2004. Cannabinoid physiology and pharmacology: 30 years of progress. *Neuropharmacol.* 47: 345-358.

Hsieh, C., Brown, S., Derleth, C., Mackie, K., 1999. Internalization and Recycling of the CB1 Cannabinoid Receptor. *J. Neurochem.* 73: 493–501.

Hu, S. S., Arnold, A., Hutchens, J.M., Radicke, J., Cravatt, B.F., Wager-Miller, J., Straiker, A., 2010. Architecture of Cannabinoid Signaling in Mouse Retina. *J. Comp. Neurol.* 518: 3848–3866.

Ientile, R., Macaione, V., Teletta, M., Pedale, S., Torre, V., Macaione, S., 2001. Apoptosis and necrosis occurring in excitotoxic cell death in isolated chick embryo retina. *J. Neurochem.* 79: 71-78.

Iversen, L., Chapman, V., 2002. Cannabinoids: a real prospect for pain relief? *Curr. Opinion Pharmacol.* 2: 50–55.

Iversen, L., 2003. Cannabis and the brain. *Brain* 126: 1252–1270.

Jakobs, T.C., Ben, Y., Masland, R.H., 2007. Expression of mRNA for glutamate receptor subunits distinguishes the major classes of retinal neurons, but is less specific for individual cell types. *Mol. Vis.*, 13: 933-948.

Katona, I., Urbán, G.M., Wallace, M., Ledent, C., Jung, K.-M., Piomelli, D., Mackie, K., Freund T.F., 2006. Molecular Composition of the Endocannabinoid System at Glutamatergic Synapses. *J. Neurosci.* 26: 5628-5637.

Keren, O., Sarne, Y., 2003. Multiple mechanisms of CB1 cannabinoid receptors regulation. *Brain Res.* 980: 197–205.

Kiagiadaki, F., Thermos, K., 2008. Effect of intravitreal administration of somatostatin and sst2 analogs on AMPA-induced neurotoxicity in rat retina. *Invest. Ophthalmol. Vis. Sci.* 49: 3080-3089.

Kozak, K., Marnett, L., 2002. Oxidative metabolism of endocannabinoids. *Prostaglandins Leukot. Essent. Fatty Acids* 66: 211–220.

Kokona, D., Charalampopoulos, I., Pediaditakis, I., Gravanis, A., Thermos, K., 2012. The neurosteroid dehydroepiandrosterone (DHEA) protects the retina from AMPA-induced excitotoxicity: NGF TrkA receptor involvement. *Neuropharmacol.* 62: 2106-2117.

Kokona, D., Thermos, K., Synthetic and endogenous cannabinoids protect retinal neurons from AMPA excitotoxicity in vivo, via activation of CB1 receptors: Involvement of PI3K/Akt and MEK/ERK signaling pathways. *Exp. Eye Res.*136: 45-58.

Kokona, D., Georgiou, P.C., Kounenidakis, M., Kiagiadaki, M., Thermos, K., 2016 Endogenous and Synthetic Cannabinoids as Therapeutics in Retinal Disease. *Neural Plastisticy* 2016: 8373020.

Kreitzer, A.C., Regehr, W.G., 2001. Retrograde inhibition of presynaptic calcium influx by endogenous cannabinoids at excitatory synapses onto Purkinje cells. *Neuron* 29: 717–727.

Ladak, N., Beishon, L., Thompson, J.P., Lambert, D.G., 2011. Cannabinoids and sepsis. *Trends in Anesthesia and Critical Care* 1(4): 191-198.

Laine, K., Jarvinen, K., Mechoulam, R., Breuer, A., Jarvinen, T., 2002. Comparison of the enzymatic stability and intraocular pressure effects of 2-arachidonylglycerol and noladin ether, a novel putative endocannabinoid. *Invest. Ophthalmol. Vis. Sci.* 43: 3216–3222.

Lax, P., Esquiva, G., Altavilla, C., Cuenca, N., 2014. Neuroprotective effects of the

cannabinoid agonist HU210 on retinal degeneration. *Exp. Eye Res.* 120: 175-185.

López, E.M., Tagliaferro, P., Onaivi, E.S., López-Costa, J.J., 2011. Distribution of CB2 Cannabinoid Receptor in Adult Rat Retina. *Synapse* 65: 388-392.

Lu, Q., Straiker, A., Lu, Q., Maguire, G., 2000. Expression of CB2 cannabinoid receptor mRNA in adult rat retina. *Vis. Neurosci.* 17: 91–95.

Maccarone, M., Van der Stelt, M., Rossi, A., Veldink, G.A., Vliegenthart, J.F., Finazzi-Agro, A., 1998. Anandamide hydrolysis by human cells in culture and brain. *J. Biol. Chem.* 273: 32332–32339.

Mackie, K., Hille, B.,1992. Cannabinoids inhibit N-type calcium channels in neuroblastoma-glioma cells. *Proc. Natl. Acad. Sci. U.S.A.* 89: 3825-3829.

Mackie, K., Devane, W., Hille, B., 1993. Anandamide, an endogenous cannabinoid, inhibits calcium currents as a partial agonist in N18 neuroblastoma cells. *Mol. Pharmacol.* 44: 498–503.

Mackie, K., Lai, Y., Westenbroek, R., Mitchell, R., 1995. Cannabinoids activate an inwardly rectifying potassium conductance and inhibit Q-type calcium currents in AtT20 cells transfected with rat brain cannabinoid receptor. *J. Neurosci.* 15: 6552–6561.

Matsuda, L.A., Lolait, S.L., Brownstein, M.J., Young, A.C., Bonner, T.I., 1990. Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature* 346: 561–564.

Mechoulam, R., Feigenbaum, J.J., Lander, N., Segal, M., Jarbe, T.U.C., Hiltunen, A.J., Consroe, P., 1988. Enantiomeric cannabinoids: stereospecificity of psychotropic activity. *Experientia* 44: 762–764.

Mechoulam, R., Ben-Shabat, S., Hanus, L., Ligumsky, M., Kaminski, N.E., Schatz, A.R., Gopher, A., Almog, S., Martin, B.R., Compton, D.R., 1995. Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. *Biochem. Pharmacol.* 50: 83-90.

Mechoulam , R., 2002. Discovery of endocannabinoids and some random thoughts on their possible roles in neuroprotection and aggression. *PLEFA* 66: 93-99.

Min, R., Nevian, T., 2012. Astrocyte signaling controls spike timing-dependent depression at neocortical synapses. *Nat. Neurosci.* 15: 746-53.

Molina-Holgado, E., Vela, J.M., Arévalo-Martín, A., Almaza, G., Molina-Holgado, F., Borrell, J., Guaza C., 2002. Cannabinoids Promote Oligodendrocyte Progenitor Survival: Involvement of Cannabinoid Receptors and Phosphatidylinositol-3 Kinase/Akt Signaling. *J. Neurosci.* 22: 9742-9753.

Molina-Holgado, F., Pinteaux, E., Heenan, L., Moore, J.D., Rothwell, N.J., Gibson, R.M., 2005. Neuroprotective effects of the synthetic cannabinoid HU-210 in primary cortical

neurons are mediated by phosphatidylinositol 3-kinase/AKT signaling. *Mol. & Cell. Neurosci.* 28: 189-194.

Morkve, S.V., Veruki M.L., Hartveit, E., 2003. Functional characteristics of non-NMDA-type ionotropic glutamate receptor channels in AII amacrine cells in rat retina. *Journal of Physiology* 542: 147-165.

Munro, S., Thomas, K.L., Abu-Shaar, M., 1993. Molecular characterization of a peripheral receptor for cannabinoids. *Nature* 365: 61-65.

Naidoo, V., Nikas, S.P., Karanian, D.A., Hwang, J., Zhao, J., Wood, J.O., Alapafuja, S.O, Vadivel, S.K., Butler, D., Makriyannis, A., Bahr, B.A., 2012. New Generation Fatty Acid Amide Hydrolase Inhibitor Protects Against Kainate-Induced Excitotoxicity. *J. Mol. Neurosci.* 43: 493–502.

Navarrete, M., Araque, A., 2010. Endocannabinoids potentiate synaptic transmission through stimulation of astrocytes. *Neuron* 68: 113–126.

Nucci, C., Gasperi, V., Tartaglione, R., Cerulli, A., Terrinoni, A., Bari, M., De Simone, C., Agrò, A.F., Morrone, L.A., Corasaniti, M.T., Bagetta, G., Maccarrone, M., 2007. Involvement of the Endocannabinoid System in Retinal Damage after High Intraocular Pressure–Induced Ischemia in Rats. *Invest. Ophthalmol. Vis. Sci.* 48: 2997-3004.

Onaivi, E.S., Ishiguro, H., Gong, J.-P., Patel, S., Perchuk, A., Meozzi, P.A., Myers, L., Mora, Z., Tagliaferro, P., Gardner, E., Brusco, A., Akinshola, B.E., Liu, Q.-R., Hope, B., Iwasaki, S., Arinami, T., Teasenfitz, L., Uhl, G.R., 2006. Discovery of the Presence and Functional Expression of Cannabinoid CB2 Receptors in Brain. *Annals New York Academy of Sciences* 1074: 514-536.

Onaivi, E.S., Ishiguro, H., Liu, Q.R., Gong, J.P., Tagliaferro, P., Brusco, A., Arinami, T., Uhl, G.R., 2009. CNS Effects of CB2 Cannabinoid Receptors. *Open Neuropsychopharmacol. J.* 2: 45-52.

Ogden, T.E., 1978. Nerve fiber layer astrocytes of the primate retina: morphology, distribution, and density. *Invest. Ophthalmol. Vis. Sci.* 17(6): 499-510.

Osborne, N. N., Casson, R. J., Wood, J. P. M., Chidlow, G., & Graham, M., 2004. Retinal ischemia: mechanisms of damage and potential therapeutic strategies. *Prog. Ret. Eye Res.* 23, 91–147.

Paes-de-Carvalho, R., Da Costa Calaza, K., Cossenza, M., Magalhães, C.R., Cabral Portugal C., Esteves da Silva Socodato R., 2008. Glutamate Receptors in the Retina: Neurochemical and Developmental Aspects. *Pages from Amino Acid Receptors Research,* Nova Science Publishers.

Pertwee R. G., 1999. Pharmacology of cannabinoid receptor ligands. *Curr Med Chem* 6: 635–664.

Pertwee,R.G., Ross R.A., 2002. Cannabinoid receptors and their ligands. *Prostaglandins, Leukotrienes and Essential FattyAcids* 66: 101-121.

Pertwee, R.G., 2006. The pharmacology of cannabinoid receptors and their ligands: an overview. *International Journal Of Obesity,* 30, 13-18.

Pertwee, R.G., Howlett, A.C., Abood, M.E., Alexander, S.P.H., Di Marzo, V., Elphick, M.R., Greasley, P.J., Hansen, H.S., Kunos,G., Mackie, K., Mechoulam R., Ross R.A., 2010. Cannabinoid Receptors and Their Ligands: Beyond CB1 and CB2. *Pharmacol. Rev.* 62: 558-631.

Porcella, A., Maxia, C., Gessa, G.L., Pani, L., 2000. The human eye expresses high levels of CB1 cannabinoid receptor mRNA and protein. *Eur. J. Neurosci.* 12: 1123-1127.

Rubino, T., Vigano, D., Premoli, F., Castiglioni, C., Bianchessi, S., Zippel, R., 2006. Changes in the expression of G protein-coupled receptor kinases and beta-arrestins in mouse brain during cannabinoid tolerance: a role for RAS-ERK cascade. *Mol. Neurobiol.* 33: 199 213.

Resnikoff, S., Pascolini, D., Mariottia, S.P., Pokharela, G.P., 2004. Global magnitude of visual impairment caused by uncorrected refractive errors in 2004. *Bulletin of the World Health Organization.* 86(1): 63-70.

Rea, K., Roche, M., Finn, D.P., 2007. Supraspinal modulation of pain by cannabinoids : the role of GABA and glutamate. *Br. J. Pharmacol.* 633–648.

Ryberg, E., Larsson, N., Sjögren, S., Hjorth, S., Hermansson, N.O., Leonova, J., Elebring, T., Nilsson, K., Drmota, T., Greasley, P.J., 2007. The orphan receptor GPR55 is a novel cannabinoid receptor. *Br. J. Pharmacol*. 152: 1092-1101.

Sánchez, M.G., Ruiz-Llorente, L., Sánchez, A.M., Díaz-Laviada, I., 2003. Activation of phosphoinositide 3-kinase/PKB pathway by CB1 and CB2 cannabinoid receptors expressed in prostate PC-3 cells. Involvement in Raf-1 stimulation and NGF induction. *Cell. Signalling*. 15: 851–859.

Savinainen, J.R., Saario, S.M., Laitinen, J.T., 2012. The serine hydrolases MAGL, ABHD6 and ABHD12 as guardians of 2-arachidonoylglycerol signaling through cannabinoid receptors. *Acta Physiol.* 204: 267–276.

Schlosburg, J.E., Blankman, J.L., Long, J.Z., Nomura, D.K., Pan, B., Kinsey, S.G., Nguyen, P.T., Ramesh, D., Booker, L., Burston, J.J., Thomas, E.A., Selley, D.E., Sim-Selley, L.J., Liu, Q., Lichtman, A.H., Cravatt, B.F., 2010. Chronic monoacylglycerol lipase blockade causes functional antagonism of the endocannabinoid system. *Nat. Neurosci.* 13: 1113-1119.

Sheng, W.S., Hu, S., Min, X., Cabral, G.A., Lokensgard, J.R., Peterson, P.K., 2005. Synthetic cannabinoid WIN55,212-2 inhibits generation of inflammatory mediators by IL-1beta-stimulated human astrocytes. *Glia* 49: 211-219.

Siliprandi R, Canella R, Carmignoto G, et al. N-methyl-D-aspartateinduced neurotoxicity in the adult rat retina. *Vis Neurosci*. 8: 567–573.

Sim-Selley, L.J., 2003. Regulation of cannabinoid CB1 receptors in the central nervous system by chronic cannabinoids. *Crit. Rev. Neurobiol.*15: 91-119.

Sim-selley, L.J., Schechter, N.S., Rorrer, W.K., Dalton, G.D., Hernandez, J., Martin, B.R., & Selley, D.E., 2006. Prolonged Recovery Rate of CB 1 Receptor Adaptation after Cessation of Long-Term Cannabinoid Administration. *Mol. Pharmacol.* 70: 986–996.

Slusar, J.E., Cairns, E.A., Szczesniak, A., Bradshaw, H.B., Di, A., Kelly, M.E., 2013. The fatty acid amide hydrolase inhibitor, URB597, promotes retinal ganglion cell neuroprotection in a rat model of optic nerve axotomy. *Neuropharmacol.* 72: 116–125.

Smith, T.H., Sim-selley, L.J., Selley, D.E., 2010. Cannabinoid CB1 receptor-interacting proteins: novel targets for central nervous system drug discovery ? *Br. J. Pharmacol.* 160: 454–466.

Stella, N., 2010. Cannabinoid and cannabinoid-like receptors in microglia, astrocytes, and astrocytomas. *Glia* 58: 1017–1030.

Straiker, A., Maguire, G., Mackie, K., Lindsey, J., 1999. Localization of cannabinoid CB1 receptors in the human anterior eye and retina. *Invest. Ophthalmol. Vis. Sci.* 40: 2442–2448.

Song, Z., Slowey, C., 2000. Involvement of cannabinoid receptors in the intraocular pressure-lowering effects of WIN55212–2. *J. Pharmacol. Exp. Ther.* 292: 136–139.

Sugiura, T., Kondo, S., Sukagawa, A., Nakane, S., Shinoda, A., Itoh, K., Yamashita, A., Waku, K., 1995. 2-Arachidonoylglycerol: a possible endogenous cannabinoid receptor ligand in brain. *Biochem. Biophys. Res. Commun.* 215: 89–97.

Sugiura, T., Waku, K., 2002. Cannabinoid receptors and their endogenous ligands. *J. Biochem.* 132: 7–12.

Sun, Q., Ooi, V.E.C., Chan, S.O., 2001. N-methyl-D-aspartate-induced excitotoxicity in adult rat retina is antagonized by single systemic injection of MK-801. *Exp. Brain Res.* 138: 37-45.

Tappe-Theodor, A., Agarwal, N., Katona, I., Rubino, T., Martini, L., Swiercz, J., Mackie, K., Monyer, H., Parolaro, D., Whistler, J., Kuner, T., Kuner, R., 2007. A molecular basis of analgesic tolerance to cannabinoids. *J. Neurosci.* 27:4165-4177.

Tsou, K., Brown, S., Sanudo-Pena, M.C., Mackie, K., Walker, J.M., 1998. Immunohistochemical distribution of cannabinoid CB1 receptors in the rat central nervous system. *Neuroscience* 83: 393– 411.

Van der Stelt, M., Di Marzo, V., 2005.Cannabinoid receptors and their role in neuroprotection. *Neuromol Med*. 7: 37–50.

Vasilaki, A., Thermos K., 2009. Somatostatin analogues as therapeutics in retinal disease. *Pharmacol. Ther.* 122: 324-333.

Wartmann, M., Campbell, D., Subramanian, A., Burstein, S.H., Davis, R.J., 1995.The MAP kinase signal transduction pathway is activated by the endogenous cannabinoid anandamide. *FEBS Lett.* 359:133-136.

Westlake,T.M., Howlett,A.C., Bonner,T.I., Matsuda,L.A., Herkenham,M., 1994. Cannabinoid receptor binding and messenger RNA expression in human brain: an *in vitro* receptor autoradiography and *in situ* hybridization histochemistry study of normal aged and Alzheimer's brains. *Neuroscience* 63: 637-652.

Willoughby, K.A., Moore, S.F., Martin, B.R., Ellis, E.F., 1997. The Biodisposition and Metabolism of Anandamide in Mice. *J.Pharmacol. Exp. Therap.* 282: 243–247.

Wilson R. I., Nicoll R. A. 2001. Endogenous cannabinoids mediate retrograde signalling at hippocampal synapses. *Nature* 410: 588–592

Yazulla, S., Studholme, K.M., McIntosh, H.H., Deutsch, D.G., 1999. Immunocytochemical Localization of Cannabinoid CB1 Receptor and Fatty Acid Amide Hydrolase in Rat Retina. *J. Comp. Neurol.* 415: 80-90.

Yazulla S., 2008. Endocannabinoids in the Retina: From Marijuana to Neuroprotection. *Prog. Ret. & Eye Res.* 27: 501-526.

Zabouri, N., Bouchard, J., Casanova, C., 2011. Cannabinoid Receptor Type 1 Expression During Postnatal Development of the Rat Retina. *J. Comp. Neurol.* 519: 1258–1280.

Zygmunt, P. M., Petersson, J., Andersson, D. A., Chuang, H., Di Marzo, V., Julius, D., 1999. Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature* 400: 452–457.